ANALYZING STABLE ISOTOPE SIGNATURES OF INORGANIC NITROGEN FORMS: A METHOD COMPARISON

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ABSTRACT

The analysis of nitrogen (N) stable isotope –signatures ($\delta^{15}N$) of ammonium (NH_4^+) and nitrate (NO_3^-) is increasingly used in biogeochemical and ecological studies to better understand processes involved in N cycling. From a methodological point of view, this analysis is not straightforward since the target N form has to be separated and purified from the other N components in the sample before $\delta^{15}N$ values can be determined. A few methods have been widely used for the N isotope analysis, most importantly the microdiffusion (MD) and chemical conversion (CM) method. In this thesis, a comparative study was conducted to reveal accuracy, reliability, advantages and disadvantages of both method and thus their applicability in ecological studies. Solutions containing NH_4^+ and/or NO_3^- at natural abundance levels were prepared and the linearity of N concentrations, stability of the $\delta^{15}N$ values over the dilution serie, recovery, the impact of the solution type (salt or water) as well as the impact of excess N on the target N on the laboratory procedure were tested by applying both MD and CM methods. It was shown that the microdiffusion of NO_3^- (NO_3-MD) was only method providing the linear increase in concentration over the dilution series. These analyses were also the only ones providing the $\delta^{15}N$ –value equal to the actual source of the tested substance, although only at the two highest concentrations. The chemical conversion of NO_3^- (NO_3-CM) resulted also in a linearly increasing concentration series, but the $\delta^{15}N$ –values tended to be too high, which was most probably an outcome of the method originally planned for other applications (labeling studies). The NH_4^+ results from MD and CM were inconsistent most probably due to the failure in the original NH_4^+ solutions as the same solution was used in both NH_4^+ analyses. The recovery-% increased by ~50 % when salt was added to the NH_4-MD when compared to a MD without salt, implying that the salt is needed in the MD analysis. Additionally, if NH_4^+ is added as an excess N-source to NO_3^-, the recovery of the N molecules is increased by ~40 % when compared to NH_4^+ -free solution, indicating the importance of decent removal of NH_4^+ before NO_3-MD. Problems which were evident in both methods were associated to lack of blank samples; the correct analysis of blank samples proved to be highly important in these analysis. In summary, both methods need development if $\delta^{15}N$ values of inorganic N forms are to be reliably determined. In this study, the MD methods resulted in higher accuracy and better ranking than the CM method.

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TIIVISTELMÄ (ABSTRACT IN FINNISH)

Typen (N) stabiili-isotooppeja ^{14}N ja ^{15}N on laajalti hyödynnetty biogeokemiallisessa ja ekologisessa tutkimuksessa mm. ammoniumin (NH_4^+) ja nitraatin (NO_3^-) suhteen. Näytteen tutkittava typpi täytyy esikäsitellä isotooppianalytiikalle soveliaaseen muotoon tarkkaa analyysia varten: N-näyte eristetään ja/tai puhdistetaan muista N:n muodoista ennen lopullista analyysia. Analyysikäyttöön on vakiintunut muutamia menetelmiä, mutta menetelmien välisiä tutkimuksia ei ole aiemmin esitetty. Vertasimme ns. mikrodiffuusion (MD) ja kemiallista kääntömenetelmän (CM) tarkkuuksia, luotettavuuksia sekä soveltuvuuksia ekologisille tutkimuksille. Vertailu tehtiin liuoksille, joissa oli NH_4^+ ja/tai NO_3^- ja joista verrattiin laimennossarjan lineaarisuutta sekä saantoprosenttia, $\delta^{15}N$-arvon lineaarisuutta laimennossarjassa sekä liuostyypin (suolaliuos tai vesi) että ylimääräisen N-muodon merkitystä (NH_4^+ ja NO_3^-). Näyteliuoksien N-isotooppikoostumukset vastasivat luonnossa esiintyviä suhteita. Mikrodiffuusio nitraatille osoittautui ainoaksi menetelmäksi, jossa mittaustuloksen konsentraatio kasvoi lineaarisesti laimennossarjan kasvaessa. Lisäksi NO_3-MD:ssa isotooppikoostumus osoittautui alkuperäistä vastaavaksi toisesta menetelmistä poiketen, mutta vain kahdella korkeimmalla pitoisuudella. NO_3-CM oli lineaarinen konsentraatiosarjan suhteen, mutta isotooppikoostumukset osoittautuivat liian rikastuneiksi johtuen käytetyn menetelmän ominaisuuksista (menetelmä on alkujaan suunniteltu rikastetuille näytteille). Ammoniumtypestä tehdyt analyysit (MD ja CM) tuottivat epäjohdonmukaisia tuloksia sekä lineaarisuuksille että isotooppikoostumuksille; syyksi tälle osoittautui todennäköisimmin epäonnistuneet näyteliuokset, joita käytettiin kummassakin kokeessa. Suolalisä NH_4^+ -näyteliuoksessa kasvatti noin 50 % saantoprosenttia verrattaessa suolattomaan liuokseen. Lisäksi, saanto kasvoi ~40 % NO_3-MD:n tehtynä NH_4:a sisältävään liuokseen verrattaessa liuokseen ilman NH_4^+:a, viitaten NH_4^+:n täydellisen poistamisen tärkeyteen NO_3^- -analyysissa. Molemmissa menetelmissä esiin tulleet ongelmat liittyivät nollanäytteiden puuttumiseen, joidenka merkitsevyys korostui mainittuja analyysejä tehtäessä. Kumpikin menetelmä vaatii kehitystä, jotta epäorgaanisen N:n $\delta^{15}N$ -arvo voidaan määrittää luotettavasti. Tässä tutkimuksessa MD-menetelmät osoittautuivat tarkemmiksi ja siten luotettavammiksi kuin CM-menetelmät.

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PREFACE

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Simo Jokinen

1. INTRODUCTION

The element nitrogen (N) has a crucial role in the biology, chemistry as well as physics of the Earth and its organisms since it is mandatory element in, e.g., nucleic acids and proteins which are basis of life. Even though dinitrogen (N_2) is the most abundant one of the atmospheric gases with the contribution of 78 %, only very scarce terrestrial or marine organisms can use it (fix it), as N_2 is very inert (e.g. Canfield et al., 2010). However, those few organisms able to fix atmospheric N_2 are responsible of most of the N allocated and stored in the aquatic and terrestrial ecosystems. As a result of N_2 fixation, N is converted to ammonium (NH_4^+) which can be taken up by plants, immobilized to microbes or fixed to negatively-charged clay minerals. In oxic conditions NH_4^+ can be oxidized (nitrified) to nitrate (NO_3^-) by chemoautotrophic bacteria. Produced NO_3^- can be taken up by plants as well, lost by leaching to aquatic ecosystems or reduced (denitrified) in anoxic conditions to gaseous N forms: nitric oxide, nitrous oxide and finally N_2-gas (NO and N_2O, respective) by soil microbes and hence be lost back to the atmosphere. Even though N is vital for life as we know it, some evident risks related to N are also known: NO destroys atmospheric ozone, leaching of NO_3^- causes eutrophication and both denitrification and nitrification has a side-product, N_2O, which is a strong greenhouse gas (Schlesinger, 1997).

Since NH_4^+ and NO_3^- have key roles in the N cycle, huge amount of research effort has been put on quantitative studies concerning their, e.g., aquatic, environmental and biogeochemical cycling. However, at current stage of N research, the origin of the N form is highlighted: how it was produced and by which processes, and finally, from which sources was it originating? These questions can be addressed with N stable isotope approaches, either by natural abundance studies or by enrichment of different N forms, so-called tracer studies. The quantities as well as qualities of the N form, its origin and further processes can be studied in detail by coupling concentration and isotope studies.

Nitrogen stable isotope samples can be analyzed with isotope ratio mass spectrometer (IRMS). Since different N forms are mainly analyzed either as N_2 or as N_2O in IRMS methods, prior to the stable isotope analysis of the specific inorganic N species, the target form of N has to be converted to analyzable form as well as purified or isolated from other N forms since they would affect the isotopic composition and hence the result. Few methods are mainly used for the N stable isotope analysis of individual components (mainly NH_4^+ and

NO_3^-). They are so-called chemical conversion method (CM), microdiffusion (MD) and microbial conversion.

Chemical method was originally used to convert inorganic N to N_2 gas for the isotope analysis (Hauck, 1982). However, as the atmosphere has high level on N_2 gas, the very high N_2-background was causing analytical problems via instrumentation leakages in analyzing of $\delta^{15}N$-N_2, especially for low N concentrations in samples. Steven and Laughlin (1994) provided CM for converting NO_3^- to N_2O. A method for NH_4^+ conversion to N_2O was established by Laughlin et al. (1997). As the above mentioned methods had limitations in recovery, Lachouani et al. published recently (2010) a new chemical method to convert NO_3^- to N_2O with upgraded recovery. In microbial method NO_3^- is denitrified to N_2O with specific microbial communities lacking the last enzyme of denitrification (N_2O reduction to N_2). Such method was described by Sigman et al. (2003). However, currently only NO_3^- can be converted with microbial conversion. Microdiffusion methods are based on conversion of inorganic N (NH_4^+ and NO_3^-) to NH_4^+-salt, and hence it is only method where sample is analyzed as a solid sample. Currently, so-called acid trap method, described e.g. by Stark & Hart (1994), is the most common MD procedure.

Most of the $\delta^{15}N$-values currently published are obtained mainly by MD methods, which is relatively easy both analytically as well as on laboratory practice. However, it is also time-consuming as well as prone to analytical errors. As the CM is getting more used, the aim of this thesis was to compare two different methods used for the $\delta^{15}N$-analysis: MD and CM. Method comparison was done with natural abundance $\delta^{15}N$ –substances with a dilution series for NH_4^+ and NO_3^-. Linearity of concentrations and isotopic compositions, recoveries as well as standard deviations was high-lighted in final method evaluation.

2. BACKGROUND

2.1 Stable isotopes of nitrogen: ^{14}N and ^{15}N

Generally, isotopes are variations of atoms which have same amount of protons but different amount of neutrons in their nuclei. As many other elements, nitrogen has two stable isotopes, ^{14}N and ^{15}N. The earth´s nitrogen pool contains 99.6337 % ^{14}N and the rest, 0.3663 %, is the other stable isotope, ^{15}N (Robinson, 2001). This difference of isotopic composition in different N pools can and has been used for, e.g., ecological applications to study the N dynamics. Analysis of N isotopes can be done by IRMS, which are commercially available.

The nitrogen isotope studies in ecology can be done on the basis of enrichment studies or by natural abundance approaches. In enrichment studies N with ^{15}N is added to a system and it can be traced back since the native N pools are of natural abundance (e.g. Hanson and Pettersson 1989, Huygens et al. 2007 and Akkal-Corfini et al. 2010). However, when excess N is added, the system is often changed from natural conditions. Studies using enrichment of isotopes are generally known as tracer studies. The dynamics of N can be also studied at natural abundance level of the N (e.g. Högberg 1997, Evans 2001 and Robinson 2001). There native N pools are studied as well as their isotopic compositions are followed. Fractionation, which is the separation of the light isotope from the heavy isotope, causes changes in isotopic composition (relative amount of ^{15}N and ^{14}N molecules in *a source and its product*) in nature. When a molecule containing e.g. N, is passing chemical, physical as well as microbial processes, the light isotope (^{14}N) will usually react faster which causes fractionation. Two types of fractionation – kinetic and equilibrium fractionation - are discussed below. Benefits of natural abundance studies are minimal disturbances to the studied systems as well as the cheaper cost.

2.2 Isotope notation

There are two different approaches to express N isotope data, depending whether tracer or natural abundances of isotopes are used; both approaches apply to the expression of other element´s isotope data as well. The expressions are δ-value and atom percent (AT-%). The δ-value is used to express the amount of naturally occurring N isotopes, while AT-percent (%) is used with tracer studies. The $\delta^{15}N$ values as well as AT-% ^{15}N abundances of isotopes are calculated, respectively, according to following calculations (Eq. 1 and 2.):

Eq. 1 $$\delta^{15}N = 1000\left(\frac{R_{sample}-R_{standard}}{R_{standard}}\right)$$

Eq. 2 $$AT-\%^{15}N = 100\left(\frac{n_{15}}{n_{15}+n_{14}}\right) = 100\left(\frac{R_{sample}}{R_{sample}+1}\right)$$

, where R is the isotope ratio (^{15}N:^{14}N) of sample and/or standard, n_{14} is the number of ^{14}N atoms in sample and n_{15} is the number of ^{15}N atoms in sample.

2.3 Fractionation

There are two main processes causing the fractionation, known as *kinetic isotope fractionation* and *equilibrium isotope fractionation*. However, as the aim of the study focuses on laboratory procedures for N isotope analysis and thus the processes are comparable to a closed system with limited substrates, the kinetic isotope fractionation is discussed more detailed.

Kinetic isotope fractionation occurs in irreversible biological, chemical and physical reactions, which are comparable to a closed system. Liberation of NH_3-gas in NH_4-MD with high pH as well as NO_3-CM, where NO_3^- is reduced to N_2O and N_2, are good examples of kinetic isotope fractionations (see 3.2.1 Microdiffusion of NH_4^+ and 3.3.2 Chemical conversion of NO_3^- to N_2O, respectively) occurring in laboratory. Chemically, lighter isotope reacts faster and physically, lighter isotope diffuses faster. This is due to the mass difference between the isotopes: higher mass has smaller kinetic energy, which is followed by smaller velocity. Smaller velocity causes decrease in diffusitivity as well as less frequent collisions with other molecules, which decreases the reaction rate (Mook et al., 2001). Higher molecular weight of the heavier isotope causes also higher binding energy between different molecules. Thereby heavier molecule needs more energy to be either combined with or separated from another molecule. However, sometimes the heavier isotope can react faster. Those reactions are known as *inverse kinetic fractionations*. Biological processes are generally irreversible and are often described by *kinetic isotope reactions*.

Equilibrium isotope fraction is reversible biological, chemical and physical reactions, which are comparable to an open system and are often temperature-dependent. For example, the CO_2-change (diffusion) between atmosphere and ocean is an example of equilibrium fractionation: CO_2 is hydrated and forms carbonic acid, which is dissociated to bicarbonate. The heaviest molecule of the reactants – bicarbonate – has the higher $\delta^{13}C$, and lower $\delta^{13}C$ is from CO_2 (Fry, 2006). The whole process is reversible and due to the equilibrium

fractionation happening between sea surface and atmosphere the value of $\delta^{13}C$ is reduced, as the heavier ^{13}C-isotopes are bound to the bicarbonate. The increasing temperature both destabilize the bonds between as their energy level increases and change the reaction rate constants thus decreasing the fractionation in a process.

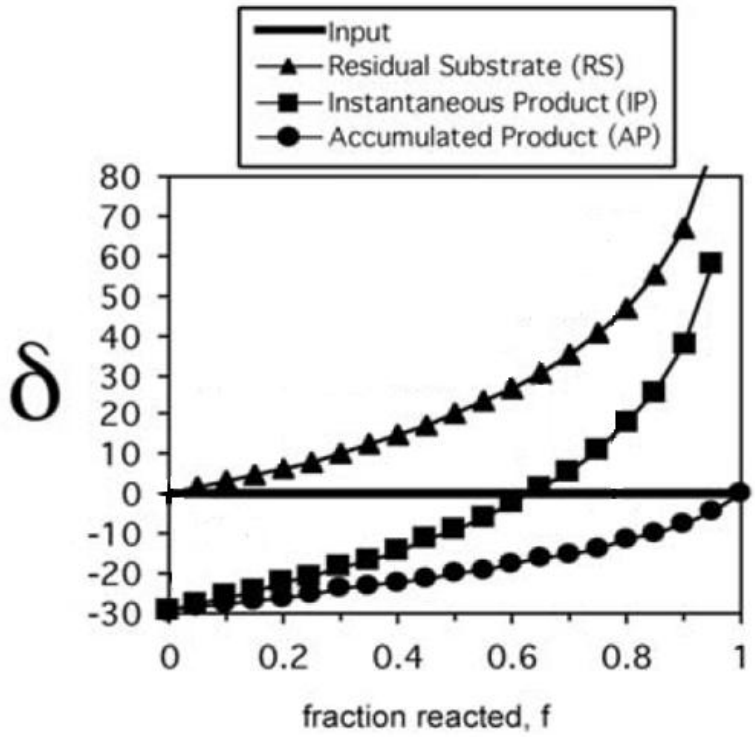

Figure 1. A schematic figure of isotope discrimination showing a theoretical conversion of a source to a product and changes of their $\delta^{15}N$ in closed system (Figure adapted from Fry, 2006).

Studied systems – either from nature or in laboratory – in which the substrate amount are not constant or endless high, are often comparable to the closed system described above and are thus often described by kinetic fractionations (Fig. 1). As shown in Fig. 1, the high recovery percent (fraction reacted) is crucial, when considering isotopic analysis. Thereby the importance of a method capable to convert most of the N to form where the isotopic composition can be analyzed is essential.

In nature, $\delta^{15}N$ is known to vary mainly from -30 to +30 ‰ (Robinson, 2001). Fig. 2 shows an example on the natural variation found from $\delta^{15}N$ in N sources contributing to the environment (http://wwwrcamnl.wr.usgs.gov/isoig/isopubs/itchfig16-4.htm). Nitrogen-containing fertilizers have the smallest deviation due to their production procedure (Haber-Bosch –process) where atmospheric N_2 - which has $\delta^{15}N$ of 0 ‰ - is fixed and converted to

fertilizers (mainly NH_4^+ and NO_3^-). The impact of the atmospheric N_2-derived fertilizer is also visible in $\delta^{15}N$ between fertilized and natural soil: although fertilized soils have scattered $\delta^{15}N$ values, the value is mostly weighted slightly above 0 ‰. Animal waste has in the Fig. 2 the scattered and most enriched $\delta^{15}N$. This is due to the fractionation of ^{15}N (or discrimination against the heavier isotope) and thus the accumulations of heavier N isotopes shown in manure, as lighter N isotopes are consumed faster.

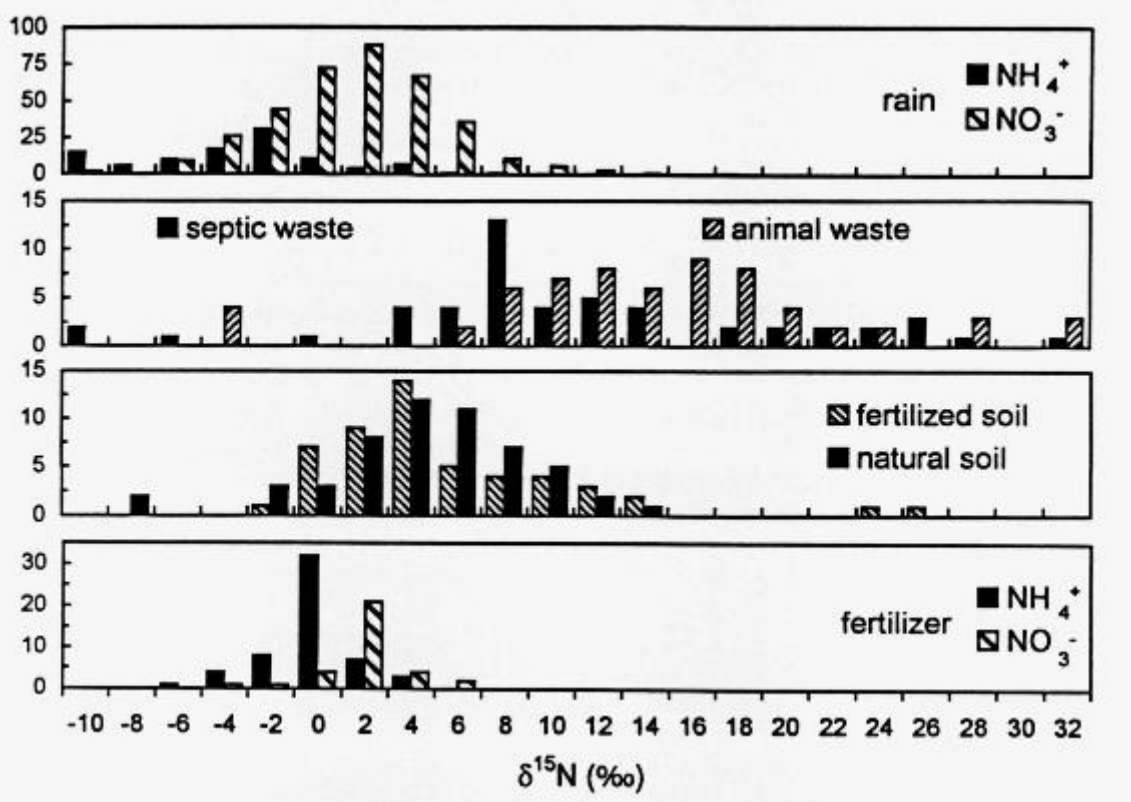

Figure 2. Variation of $\delta^{15}N$-value of the major N sources in the hydrosphere and terrestrial environment (Figure adapted from http://wwwrcamnl.wr.usgs.gov/isoig/isopubs/itchfig16-4.html)

2.4 Rayleigh model

Both kinetic and equilibrium isotope fractionations are commonly modeled using Rayleigh equations. In theory, isotope fractionation process – both kinetic and equilibrium isotope processes - can be simplified to occur either in closed system (Fig. 1) or open system. The input of a substance is limited in a closed system, while it is endless in open system. Classical Rayleigh fractionation occurs when a substrate mass is depleted during a physicochemical reaction and a product is removed from a system (closed system). As the conversion of a substrate to a product starts, more depleted (lighter) molecules react faster and turn to product in a closed system (Fry, 2006). Simultaneously the remaining substrate molecules are

enriching due to the absence of the lighter molecules (which are already products); the relative amount of enriched (heavier) molecules is increasing. A natural logarithmic enrichment is found from the substrate molecules, as the reaction proceeds. Instantaneous products are in a transition stage. When reaction is completed, the product achieves δ-value of the original input. The equation describing Rayleigh process is:

Eq. 3 $$R_{t1} = R_{t0} f^{(1-\alpha)}$$

, where R_{t0} and $R_{t1}$are the isotope ratios at different times (t_0 and t_1), f is the fraction remaining when $t = t_1$ and α is fractionation factor.

In the open system, where the input of a source is endless, the fractionation stays constant due to the steady input. Reaction is linear between substrate and product. When the substrate availability is considered as an infinite reservoir (*f* is close to 0), then the fractionation factor from product to substrate can be approximated according to following equation:

Eq. 4 $$\Delta = \delta_{source} - \delta_{product}$$

, where Δ is fractionation factor, δ_{source} is the delta-value of the source and $\delta_{product}$ is the delta-value of the product.

2.5 Analysis of $\delta^{15}N$ from gas and solid samples with isotope ratio mass spectrometer

Correct laboratory practices and measurement analytics are critical when measuring the isotope values of substrates and products as well as fractionation factors of processes. Chemical conversion methods produce N_2O and MD methods produce NH_4^+ -salt of the inorganic N (here NH_4^+ and NO_3^-). Gases and solid samples have different analysis methods while the final $\delta^{15}N$ can be analyzed with the same device (IRMS).

The $\delta^{15}N$ of N_2O is analyzed with IRMS coupled to pre-concentration unit (Precon) and gas chromatograph (GC; the whole analytical system is often abbreviated as Precon-GC-IRMS). In order to achieve a proper N_2O analysis with Precon-GC-IRMS, N_2O has to be purified from CO_2 which has same molecular weight [mass is 44, 45 or 46, depending on the amount of neutrons in N (or C)]. In short, N_2O from the sample is first purified of excess CO_2 and H_2O with a chemical trap [containing ascarite (NaOH) for CO_2-removal and $Mg(ClO_4)_2$ for H_2O removal], then concentrated with liquid N_2 traps and finally separated from other gases in a GC column. GC-separated sample gas is finally directed to IRMS with He-carrier gas,

where the different masses of N_2O (44, 45 and 46) are quantified. Analysis procedure is adapted from Brand (1995).

The $\delta^{15}N$ of NH_4^+ is analyzed with an elemental analyzer (EA) coupled to IRMS (EA-IRMS) in the form of filter papers where NH_4^+ is trapped (filter trap, "solid sample"). Solid sample is first oxidized to gaseous form in high temperature (~900 ^oC) and then reduced at ~680 ^oC to N_2 which is finally directed to IRMS with He-carrier gas, where the different masses of N_2 (masses 28, 29 and 30) are quantified. This procedure has long and routinely been used in stable isotope analytics.

Estimated costs for one sample, taking into account consumables needed, are for both EA-IRMS and Precon-GC-IRMS analysis around 4 - 5 €. The main difference comes from the required analysis time of samples by different approaches: one MD-sample analyzed with EA-IRMS lasts about 8 minutes while one CM-sample analyzed with Precon-GC-IRMS lasts about 37 minutes with the current settings at Biogeochemistry laboratory at UEF. Thus, analysis of e.g. 100 samples last a bit more than 13 h with EA-IRMS while Precon-GC-IRMS needs 62 h for the mentioned sample amount, corresponding to almost five times longer time.

2.5.1 Standards in IRMS analysis

As with other spectrometers, IRMS analysis needs calibrated standards for determining correct isotope values. While, e.g., in gas chromatography the standardization is based on a known area (peak size) of a known sample [known concentration (ppm)], IRMS analysis is based on a known isotopic value of the standard (here $\delta^{15}N$). Results obtained by IRMS are related finally to the $\delta^{15}N$ value of a known standard.

In excess to internal standards, IRMS needs also a well-known reference standard (gas), which has – after it has been defined - a fixed value. These reference gases for $\delta^{15}N$ analysis are pure N_2 (99.999 %) for EA-IRMS and N_2O (99.000 %) for Precon-GC-IRMS. As sample is analyzed either as solid or gaseous, the sample peak obtained is compared to these reference gases. Usually three reference gas peaks are introduced to the IRMS prior the actual sample; these are introduced for each individual sample. The $\delta^{15}N$ of the sample is calculated with the known $\delta^{15}N$ of the reference gas by Isodat 2.82, which is the program used to operate IRMS. However, as the reference gases are directly injected to the IRMS and are thus not subjected to sample treatments in the IRMS analysis procedure, internal standards are needed. Internal standard for $\delta^{15}N$ analysis used at University of Eastern Finland, Biogeochemistry

laboratory, is prolin saccharose (PSS) with $\delta^{15}N$ of -8.67‰. Depending on the amount of the sample N, the PSS standard is chosen by the N amount to achieve sample range for EA-IRMS. Internal standard for $\delta^{15}N$ analysis of N_2O with a Precon-GC-IRMS is 800 ppb N_2O standard gas (AGA, compressed gas and balanced to synthetic air) with $\delta^{15}N$ of 8.83 ‰. Principally, a precision of 0.5 ‰ should be achieved with isotope analysis (five internal standards). Basically, any homogenous substance, which $\delta^{15}N$ is known (analyzed against a known standard, e.g. USGS or IAEA standards), can be used as a standard.

A minimum of three internal standard samples are added to the beginning of a sequence of IRMS analysis because their N content as well as N isotopic composition are well-known, and thus the instrument precision is shown prior to running of actual samples. These standards are also "fixed" for the calculations. Blank samples (e.g. empty tin cup for EA-IRMS, pure N_2 for Precon-GC-IRMS) are also added to the sequence to show the background of the instrument (e.g. leakages). Standards and blanks are added between 5 to 10 samples to keep track, whether something unexpected occurs during sample sequence, or to indicate the instrument "drift" which can subsequently be used for correcting the real samples for the drift. The drift-correction is based on the frequent standard samples in a sequence: the possible change/difference between two standards in a sequence can be mathematically noted in the samples between these standards.

2.5.2 Blank samples and blank correction

When preparing laboratory samples for IRMS analysis, blank samples must be prepared, as the solvent, used chemicals (either by purpose or by accidental contamination) and/or dishes used might contain the final target N form. Hence, a pure original solvent must be carried over the steps of the sample treatments and finally analyzed with samples, to show possible contamination due to the impact of the solvent, reagents and laboratory practices conducted. This has crucial importance when assessing isotopic composition as well as the concentration of a sample. Nevertheless, in the protocols we adopted here, no blank samples were included in the tested methods (as discussed further on).

When sample is analyzed with IRMS, the analyst (here sample N) possibly contains in excess to the target N some contaminants coming from the reagents (blank N). The impact of the blank-N in the sample-N can be deducted by so-called *blank correction*. The remaining N fraction in the equation ($\delta_{source1}$) is the target N. After the drift correction (see 2.5.1 Standards in IRMS analysis) a two-pool mixing model can be used for the blank correction:

Eq. 5 $$\delta_{sample} = \delta_{source1} * f_1 + \delta_{source2} * f_2$$

, where δ_{sample} is the drift-corrected measured $\delta^{15}N$ value of the sample (contains both target N and blank N and thus equals to 1), $\delta_{source1}$ is the $\delta^{15}N$ value of the target N (unknown), f_1 is the amount (fraction) of target N, $\delta ource_2$ is the drift-corrected $\delta^{15}N$-value of a blank and f_2 is the amount (fraction) of the blank; the fractions $f_1 + f_2 = 1$ in the two-pool mixing model. Thus, the $\delta_{source1}$ can be calculated.

2.6 Background of the two methods under evaluation

Both methods evaluated here aim to separate and produce inorganic N forms from a mixture, which can be analyzed with IRMS. Microdiffusion produces N-salt and CM produces N_2O gas. However, these methods described here are not the original versions when discussing about methods to analyze $\delta^{15}N$ of inorganic N. Below is a short summary of the history of the evaluated methods as well as method descriptions.

2.6.1 Microdiffusion of inorganic N to NH_4^+-salt

The first versions of separating inorganic N forms for analyzing them as a "solid sample" (inorganic N forms trapped to filter paper) with EA-IRMS were based on steam distillation according to Stephan and Kavanagh (2007) and references therein. As the steam distillation needed high temperatures, there were strong potential for fractionation. Distillation techniques were soon followed by diffusion methods, generally later known as *microdiffusion* (MD) (Stephan et al., 2007). The principle of the MD was to convert inorganic N from liquid phase to N-containing salt with acid and hence also concentrate the target N (Stark & Hart, 1996). This can be done from alkaline solution containing NH_4^+. When pH is reaching 9, NH_4^+ is reduced to gaseous NH_3, which can be then trapped (diffused) by acid (e.g. $KHSO_4$). This N-containing acid trap can be further analyzed with EA-IRMS for its $\delta^{15}N$-value. Method is easily applicable for NO_3^- as well: a strong redactor (Devarda´s alloy) is used to reduce NO_3^- to NH_4^+, which is then further treated as NH_4^+ in MD method.

2.6.2 Chemical conversion of inorganic N to N_2O

First chemical conversions of inorganic N conversion to gaseous N to analyze the $\delta^{15}N$ were done by Hauck (1982), who converted inorganic N (NH_4^+ and NO_3^-) in alkali conditions to NH_4^+-salt with acid. By adding NaOBr to the NH_4^+-salt, NH_4^+ was reduced mainly to N_2, but N_2O was produced as a side-product at low concentrations (1.5 to 3.0 %). The produced N_2–

gas was analyzed for its ^{15}N-content. However, N_2-analysis had serious problem due to the background-ambient air N_2 with contribution of 78 %, which is a challenge for instrument leakages. The problem of high N_2-background was solved by Stevens et al. (1993) when they introduced IRMS with pre-concentration unit (Precon-GC-IRMS) capable to, e.g., N_2O analysis. The ^{15}N-N_2O –analytics was then developed rapidly by Stevens & Laughlin (1994), who provided a method for NO_3^- conversion to N_2O by reduction of NO_3^- in acidic conditions, where reduction intermediates contain N_2O as well, with a mentioned recovery of ~10 %. With the possibility to analyze N_2O, the side product from the method described by Hauck (1982) was modified to use for NH_4^+ -samples. Laughlin et al. (1997) optimized the method described by Hauck (1982), with the increment of recovery from earlier 1.5 – 3.0 % up to 25 %. We tested here the original, earlier versions of these protocols where N_2O is only a side product.

2.7 Aim of the thesis

The aim of this thesis was to test and compare two different methods used for isotope analysis of inorganic N forms (NO_3^- and NH_4^+). Tested sample preparation methods were CM and MD and the sample analyses were done with Precon-GC-IRMS and Conflo-EA-IRMS, respectively. Chemical conversion had different approaches for NH_4^+ and NO_3^-; NH_4^+ -conversion was adapted from Hauck (1982) and Saghir et al. (1993); a procedure of Steven & Laughlin (1994) was followed for NO_3^-convertion. A procedure of Stark & Hart (1996) and references therein - later used by e.g. Biasi et al. (2005) - was used for MD of both NH_4^+ and NO_3^-. The chemical method was for this thesis for the first time applied in the laboratories of Biogeochemistry (UEF), while MD is in practice since several years, however, only for labeled samples. Method comparison was made of samples prepared from $NaNO_3$ and $(NH_4)_2SO_4$ reagents at natural abundance levels of N. A dilution series from 7 to 500 µmol N L^{-1} were made of both reagents in order to follow the recovery-%, test for shifts in isotopic composition based on variable concentrations and the isotopic composition produced by different sample preparation methods as well as IRMS analysis methods.

3. MATERIALS AND METHODS

3. 1 Experimental set up

The following parameters were comparatively assessed by using the two methods:

1) Linearity of N concentration in a dilution series.
2) Stability of $\delta^{15}N$ over a dilution serie as well as accuracy of the $\delta^{15}N$ (it should be same as the original source).
3) Comparison of solution types used for the analysis: deionized water (milli-Q-H_2O) and 1 M potassium chloride (KCl) solution
4) Impact of other N species on concentration and isotopic signature of target N component

Sample solutions were prepared from a stock solution made from $NaNO_3$ (J. T. Baker, batch 991171 0010) and $(NH_4)_2SO_4$ (J. T. Baker, batch 990423 0045) by dissolving reagents to deionized water and preparing the dilution series as indicated in Table 1 (tested parameters 1 and 2 from list above). The stock solution hence contained both inorganic N forms. Solution concentrations were designed to be realistic: highest concentrations were comparable to those found from a mineral soil under agricultural practices in Finland (Maaninka: Jokinen et al., unpublished data). Each sample was analyzed in replicates of four.

Table 1. List of samples for dilution series to test recovered N concentration and $\delta^{15}N$ values (tested parameters 1 and 2). Abbreviations: CM corresponds to chemical conversion method, MD corresponds to microdiffusion method.

Method	**IRMS Analysis**	**Concentration (µmol NH_4 L^{-1} and µmol NO_3 L^{-1})**						
		7	**15**	**30**	**60**	**125**	**250**	**500**
NH_4-CM	Precon-GC-IRMS	4	4	4	4	4	4	4
NO_3-CM	Precon-GC-IRMS	4	4	4	4	4	4	4
NH_4-MD	EA-IRMS	4	4	4	4	4	4	4
NO_3-MD	EA-IRMS	4	4	4	4	4	4	4

Solutions containing only one of the inorganic N forms (Table 2), were prepared with concentration of 0.5 mM to study, whether other N form would have impact on the target N (tested parameter 3). As NH_4^+ is often extracted with 1 M KCl, NH_4^+ -salt was additionally

dissolved in 1 M KCl in order to study the difference between water and 1 M KCl as an NH_4^+ solvent (tested parameter 4).

Table 2. List of samples for solution comparison and impact of additional N species (tested parameters 3 and 4) Abbreviations: CM corresponds to chemical conversion method, MD corresponds to microdiffusion method.

Methods	**Sample preparation**	
	Inorganic N	**Solution**
NH_4-CM, NO_3-CM NH_4-MD, NO_3-MD	NH_4^+ and NO_3^-	milli-Q-H_2O
NH_4-CM, NH_4-MD	NH_4^+	milli-Q-H_2O
NH_4-CM, NH_4-MD	NH_4^+	1 M KCl
NO_3-CM, NO_3-MD	NO_3^-	milli-Q-H_2O

3.2 Microdiffusion of NH_4^+ and NO_3^-

A procedure of Stark & Hart (1996) was adapted for MD for both NH_4^+ and NO_3^- (for detailed reaction mechanisms as well as equipments needed, see Appendix 1.1, 1.2 and 1.3, respectively).

3.2.1 Microdiffusion of NH_4^+

A 10 ml solution containing NH_4^+ (as single species or in combination with NO_3^-) was transferred to a 120 ml infusion bottle. After addition of 0.1 g of magnesium oxide (MgO) and acid trap the bottle was immediately closed with a septum stopper and a tightening ring immediately. Sample bottle was kept in a heated shaker (+ 35 ^oC, 150 rpm) for the next 4-5 days to allow the reactions (diffusion of liberated NH_3 to the acid trap) to finish.

3.2.2 Microdiffusion of NO_3^-

A 10 ml solution containing NO_3^- (as single species or in combination with NH_4^+) was transferred to a 120 ml infusion bottle. An equal amount of 4 M KCl and 0.1 g of MgO was added to the solution to make it alkaline. After the mentioned additions the sample bottle was kept in a shaker (150 rpm, 4 h) without septum stopper to allow liberation of the formed NH_3 to the atmosphere. This step is necessary to remove the possibly existing NH_4^+ from the background before conversion of NO_3^- to NH_4^+. After NH_4^+ removal, 0.5 g of Devarda´s reagent and acid trap were added to the sample bottle. Devarda´s reagent is a strong reducing agent, which reduces NO_3^- to NH_4^+ in alkaline conditions. Since reactions start immediately,

the glass bottle was closed with a septum stopper and a tightening ring immediately. Sample bottle was kept in a heated shaker (+ 35 ^{o}C, 150 rpm) for the next 4-5 days to allow the reactions (diffusion of liberated NH_3 to the acid trap) to finish.

3.2.3 Post-processing of acid traps

When MD (either NH_4^+ or NO_3^-) was finished, the infusion bottle was opened and the acid trap was carefully removed. The acid trap was dried softly and stored in a 2 ml Eppendorf – vial, which was then placed with its stopper open into a desiccator. A decanter glass with 30 ml of concentrated sulphuric acid (>97 % H_2SO_4) was held inside the desiccator, and vacuum was created into the desiccator with a water flow. The acid solution starts to boil in decreased pressure and sulphuric atmosphere is formed into the desiccator. Sulphuric atmosphere dries acid traps and stabilizes the formed NH_4^+ -salt inside the traps. Samples are kept 24 h in the sulphuric acid atmosphere for drying.

After drying the acid trap samples were opened carefully with tweezers. Filter discs were placed into tin cups, which were closed. Samples (acid traps) inside the tin cups were ready for analysis by EA-IRMS for δ^{15}N-NH_4^+ or δ^{15}N-NO_3^-.

3.3 Chemical conversion of NH_4^+ and NO_3^- to N_2O

The chemical conversion methods used had different approaches for analyses of NH_4^+ and NO_3^-: NH_4^+ -conversion was adapted from Hauck (1982) and Saghir et al. (1993) and NO_3^- conversion was adapted from Steven & Laughlin (1994). More detailed reaction mechanisms as well as laboratory procedures are found from Appendix 2.1 and 2.2, respectively.

3.3.1 Chemical conversion of NH_4^+ to N_2O

A 50 ml solution containing NH_4^+ (as single species or in combination with NO_3^-) was transferred to a 500 ml infusion bottle and 0.2 g of MgO was added to the bottle to increase alkalinity of the solution. A vial (16*100 mm) containing 3 ml of H_2SO_4:$CuSO_4$ solution (Appendix 2.1) was carefully inserted into the infusion bottle. Infusion bottle was closed tightly and put on a shaker (50 rpm) for 24 hours to allow the liberation of NH_4^+ as NH_3 and further absorbance of NH_3 to the acid inside the vial. After finished reactions the vial containing absorbed NH_4^+ was dried in an oven at 150 °C for excess water removal. Then the vial was sealed with a septum, evacuated and helium-flushed (He) and finally left with 1 bar of He to keep the vial headspace free of ambient N. Then 1 ml of NaOBr was injected to the

bottom of the vial with a long needle and the vial was left to stand for five minutes to allow the reaction between NaOBr and $H_2SO_4/(NH_4)_2CuSO_4$ –salt to produce N_2O. Produced N_2O was collected by using two 20 ml syringes, which were connected to the vial with needles: 2 M KCl was injected slowly to the vial by other syringe while the second needle was used to collect the head-space gas. As KCl-solution was injected, the produced gas from the headspace was pushed to second, empty syringe. Collected gas was then transferred from the syringe to an evacuated vial and the sample was ready for analysis with Precon-GC-IRMS for $\delta^{15}N\text{-}N_2O$.

3.3.2 Chemical conversion of NO_3^- to N_2O

A 50 ml solution containing NO_3^- was transferred to a 120 ml infusion bottle. An aliquot of 2.5 ml of 0.2 M sulphamic acid was added to the bottle to decrease the pH to 1.7. The bottle was closed and shaken for 5 seconds to reduce the existing nitrite (NO_2^-) to N_2. The bottle was then opened (N_2 released to the atmosphere) and pH was increased to 4.7 by adding 5 ml of acetate buffer solution. Prepared Cd/Cu reductor (see Appendix 2.2) was added to the bottle, which was immediately closed and put to a shaker (120 rpm) for 2 h. After shaking 12 ml headspace gas sample was taken from the bottle to evacuated vial. Samples were analyzed for $\delta^{15}N\text{-}N_2O$ with Precon-GC-IRMS.

3.4 Analysis of $\delta^{15}N$ of the samples – isotope ratio mass spectrometry

Both solid and gaseous samples were be analyzed with IRMS available at the University of Eastern Finland. Solid samples were analyzed with EA coupled to IRMS (EA-IRMS) and gaseous samples (here N_2O) were analyzed with pre-concentration unit and gas chromatograph (Precon-GC-IRMS) (see 2.5 Analysis of $\delta^{15}N$ from gas and solid samples with isotope ratio mass spectrometer). Analysis procedures as well as used standards used for both analyses were explained in chapter 2.5.1 (Standards in IRMS analysis). Elemental analysis needs ash-purification of reactors between ~100 samples and new reactors (oxidation and reduction) after ~500 samples as well as chemical CO_2 and H_2O trap. For comparison, one chemical trap (as with EA-IRMS: CO_2 and H_2O are trapped) lasts couple of thousands of samples in Precon-GC-IRMS. Additionally liquid N_2 is needed for each sample in Precon-GC-IRMS for N_2O trapping (concentrating).

3.4.1 Analysis of the original standard substances

Original salts used for the sample solutions [$NaNO_3$ and $(NH_4)_2SO_4$] were additionally weighted in to tin cups for analysis of their isotopic composition. The EA-IRMS needs about 0.01 mg N for $\delta^{15}N$ analysis; three replicates of both substances were prepared. Analysis was done with EA-IRMS.

3.5 Calculations

3.5.1 Calculation of recovery for chemical conversion

The raw results on N concentrations received from the instruments (Precon-GC-IRMS and GC-ECD for CM and EA-IRMS for MD) were first calculated to ppm N_2O or µg N, respectively, by comparing areas with simultaneously analyzed internal standard with known concentrations (0.8 ppm of N_2O standard gas for both GG-analyses, 0.043 mg N of PSS for EA-IRMS analysis). Since the output of CM and MD were different, all products were calculated to µmol N L^{-1} in order to achieve comparable dilution series as well as recovery percentages. Therefore, the results of the chemical methods (initially ppm N_2O) were calculated with the ideal gas law to the mass unit (µg N_2O):

Eq. 6 $$pV = nRT \; and \; n = \frac{m}{M} \rightarrow m = \frac{MpV}{RT}$$

, where m was mass of N_2O (g), M was mole mass of N_2O (44.01 g mol^{-1}), p was pressure (1.01325 bar), V was headspace volume of the vessel where chemical conversion was conducted (0.50 dm^{-3} for NH_4^+ conversion and 0.12 dm^{-3} for NO_3^- conversion), R was gas constant (0.08315 bar $dm^{-3}mol^{-1}$ T^{-1}) and T was temperature (294.15 K).

The gained mass was calculated to c (µmol N_2O L^{-1}) and finally to n (µmol N) by following equations:

Eq. 7 $$n = \frac{m}{M};\; c = \frac{n}{V} \rightarrow c = \frac{m}{(M*V)}$$

Amount n (µmol N) was used to achieve recovery percentages by following equation (Eq. 7):

Eq. 8 $$\text{Recovery} - \% = \frac{\text{n(product)}}{\text{n(source)}} \times 100$$

Since the chemical methods produce N_2O, the CM products are multiplied by two to note the two N atoms in *n*.

3.5.2 Calculation of recovery for microdiffusion

Microdiffusion results from EA-IRMS were calculated to mass (µg N) with known internal standards (PSS). The gained mass was calculated to c (µmol NO_3 L^{-1} or µmol NH_4 L^{-1}) and finally to n (µmol N) with the equation 7, where m was mass of NH_4^+ or NO_3^- (g) of a sample, M was mole mass of NH_4^+ or NO_3^- (18.04 g mol^{-1} and 62.01 g mol^{-1}, respectively) and V was solution amount in the vessel (0.10 L for MD). Recovery-% was calculated according to the Eq. 8.

3.5.3 $\delta^{15}N$ of the product

As explained in the chapter 2.5.1 (Standards in IRMS analysis), the achieved results for both CM and MD analyses were first corrected for the drift with frequently added known standards in the sequence.

Samples were corrected for the blank (see 2.5.2 Blank samples and blank correction) after the drift-correction. However, as the blank samples were not added to the laboratory procedures, an arbitrary blank N ($\delta^{15}N$-value and amount N) was later on included in each method for the blank correction. The arbitrary values were based on the earlier analyses of each method.

4. RESULTS

4.1 Linearity of nitrogen concentration over the dilution series

The linearity of the dilution series (concentration) of the different methods are shown in Figs. 3 A – D. Nitrate samples had good linearity for both tested methods (Figs. 3 B and D, r^2 = 0.9724 and 0.9991, respectively), except for the highest (500 µmol NO_3 L^{-1}) concentration analyzed with CM, which was slightly below the linear curve. The standard deviations of the replicate analysis tended to increase with increasing concentration with both methods for NO_3^-.

While the linearity of dilution was good for NO_3^- samples, the relationship between the NH_4^+ concentration in the samples and the products was not optimal (Figs 3A and 3C). While there should have been a linear increase over the dilution series, both of the NH_4^+-methods showed a non-linearity (or curvilinearity), with lower concentrations measured in the middle range. For CM, N_2O concentration analyses were done additionally with a gas chromatograph [gas chromatograph - electron capture detector (GC-ECD); Agilent 6890N] to evaluate quantitative results of IRMS (data not shown). The N_2O concentrations analyzed with GC-ECD had similar trend in linearity than with Precon-GC-IRMS, suggesting problems in the solution analyzed, or in the dilution of the samples (GC-ECD data not shown).

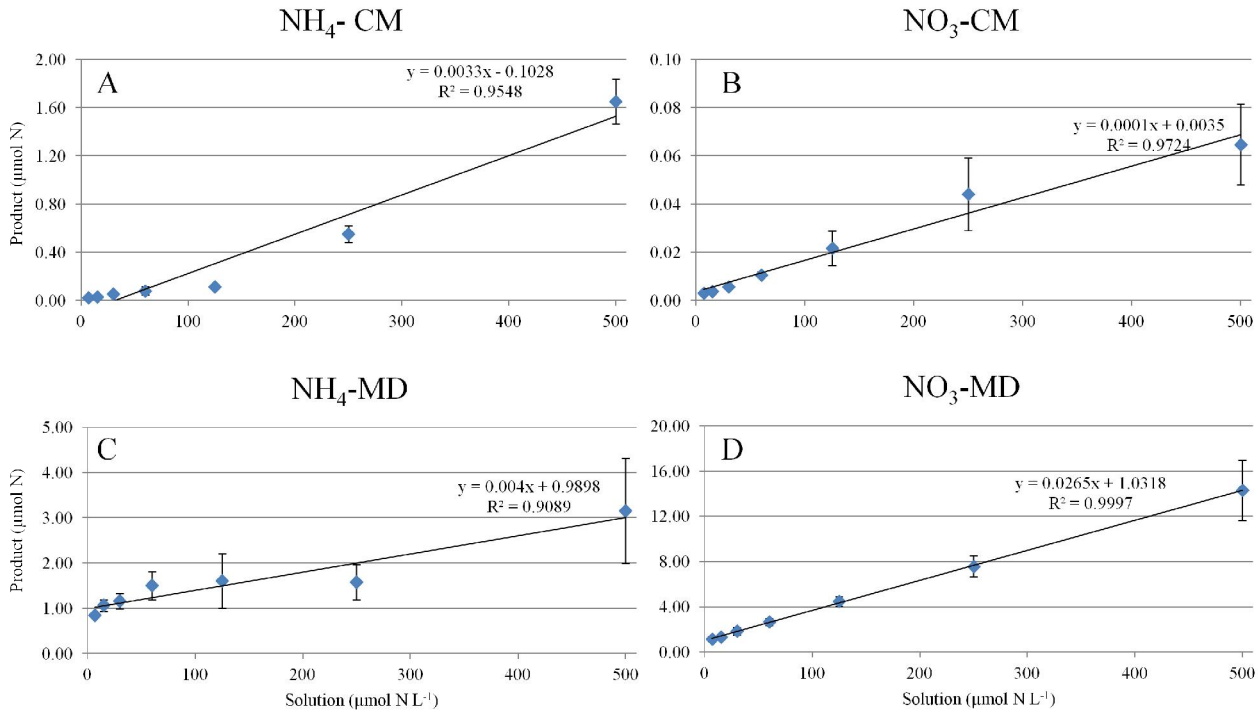

Fig. 3. The concentrations (µM N) of the dilution series with the different methods used. Note the differences in y-axes. Error bars indicate standard deviation. Abbreviations: CM corresponds to chemical conversion method, MD corresponds to microdiffusion method.

4.2 Nitrogen recoveries

The recovery of N tended to decrease logarithmically with increasing concentrations (Fig. 4 A, B) with both N forms and both methods. Ammonium prepared with CM was the only exception, where the N-recovery first decreased, but then started to increase again after 125 µmol N L^{-1}.

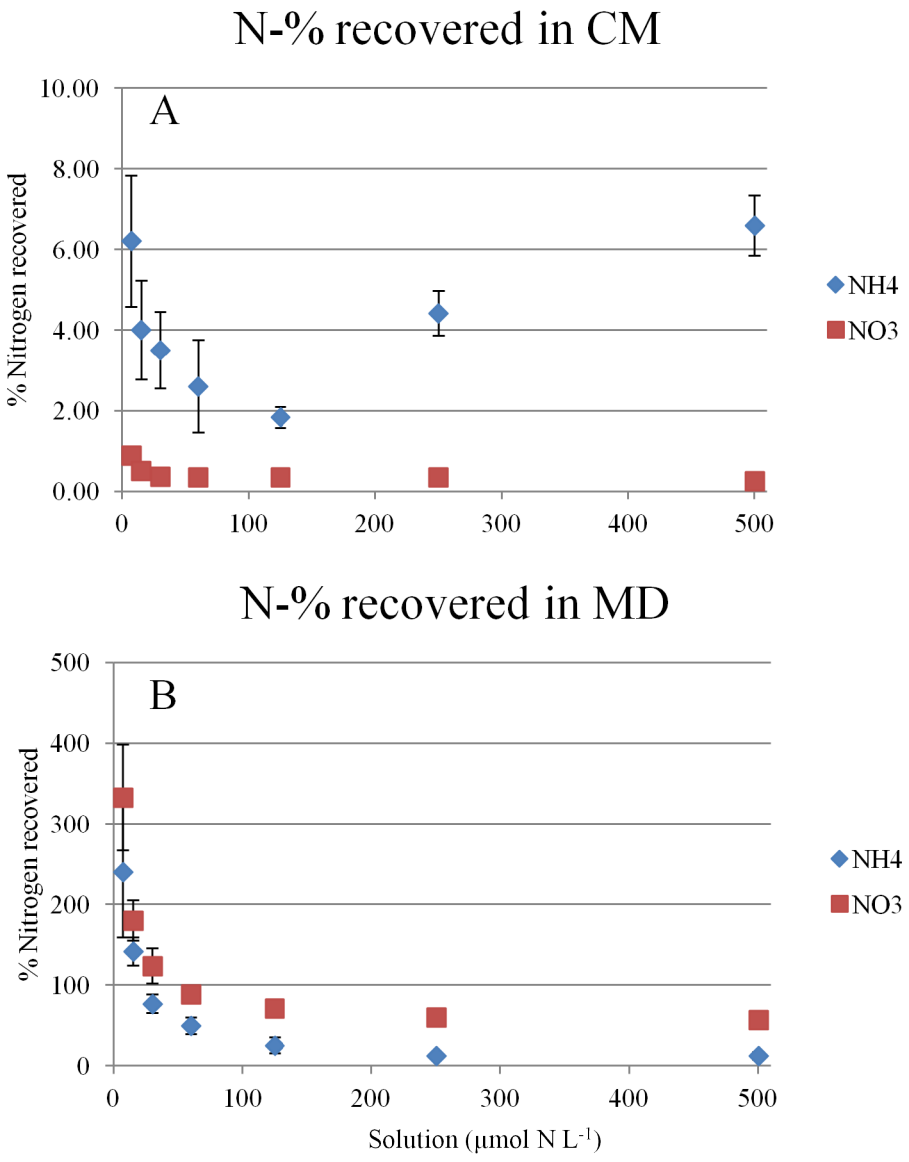

Figure 4. Recovery percentages of nitrogen analyzed with different methods. Note the difference in scales in y-axes. Error bars indicate standard deviation. Abbreviations: CM = chemical method and MD = microdiffusion.

The magnitude of recovery was completely different between CM and MD methods. The CM methods had clearly less recovered N than MD. The recovery of NH_4^+ analyzed with CM ranged between 1.8 and 6.6 %, while NO_3-CM ranged between 0.3 and 0.9 %. Both MD dilution series had logarithmically decreasing recoveries. The recovery of NH_4^+ analyzed with MD ranged between 13 and 280 % and for NO_3^- between 57 and 330 %. Lower concentrations (7 – 15 µmol NH_4 L^{-1} for NH_4^+, 7 – 30 µmol NO_3 L^{-1} for NO_3^-) had higher than 100 % recovery in MD analysis, which is more than the amount available from the original sources. Most likely this was due to the lack of blanks in the study (see discussion) and hence due to calculation problem.

4.3 Linearity of $\delta^{15}N$ over the dilution series

A logarithmic decrease of $\delta^{15}N$ of produced N with increasing concentration was found with each method except NH_4^+ prepared with MD, where the trend was opposite (Fig. 5). The original standard substances [$(NH_4)_2SO_4$ and $NaNO_3$] had slightly negative $\delta^{15}N$ -values (-0.17 ± 0.46 ‰ and -0.88 ± 0.27 ‰, respectively).

Ammonium samples analyzed with CM had more depleted $\delta^{15}N$ values in the raw data than NO_3^- (raw data; see Fig. 5 A and B). The $\delta^{15}N$ value of N_2O in NH_4-CM decreased from 10.7 to – 8.7 ‰, thus a shift of 19.4 ‰ in total. For NO_3-CM a drop from 20.2 to 5.9 ‰, corresponding to a decrease of 14.4 ‰ in total, was observed. Analysis of NO_3^- had hence smaller change in the $\delta^{15}N$ value than NH_4^+ in general, but both methods had nevertheless large shifts.

Ammonium samples prepared with MD varied from 6.5 to -3.4 ‰ on average, with no clear tendency (a max. shift of 9.9 ‰) (raw data; Fig. 5 C). In the middle range of concentration dilutions (60, 125 and 250 µmol NH_4 L^{-1}) relatively stable $\delta^{15}N$ of ~ 6 ‰ values were observed in the products. In the NO_3-MD the $\delta^{15}N$ values decreased on average from 7.9 to 1.5 ‰ (shift of 6.4 ‰ in total; Fig. 6 D).

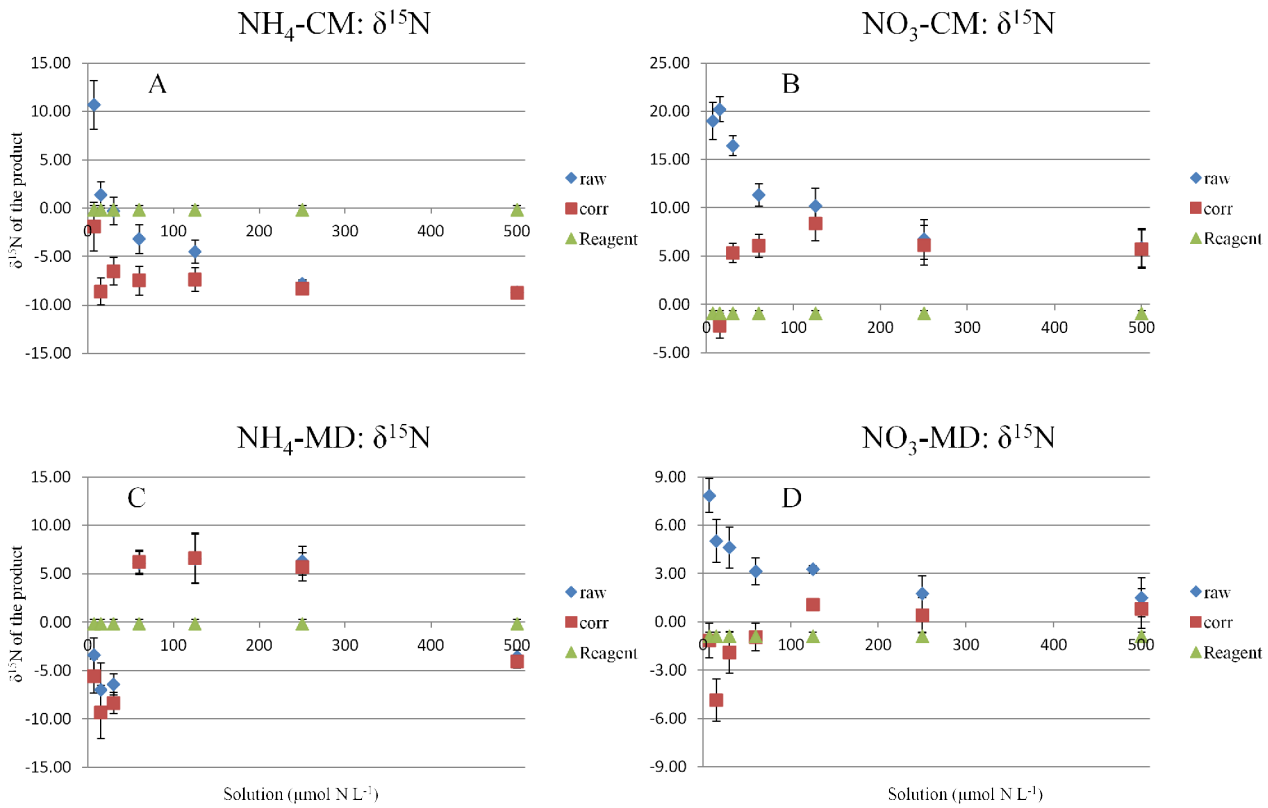

Figure 5. The $\delta^{15}N$ values of the dilution series with the different methods used. Blue diamonds indicate the raw data, red squares indicate the corrections with arbitrary blank-values, and green triangles indicate the original substrate analyzed with EA-IRMS as a salt. Note the differences in y-axes. Error bars indicate standard deviation. The smallest corrected arbitrary blank-value in the figure B (NO_3-CM) was deleted as it was -56 ‰.

4.3.1 Arbitrary blank correction

Red squares shown in the Fig. 5 indicate the arbitrary blank-corrected $\delta^{15}N$ values. As the raw data (blue diamonds) shows, a logarithmic decrease of $\delta^{15}N$ of produced nitrogen occurs with increasing N concentration. This is due to the impact of the blank (background contamination) existing in each sample. In blank correction the fraction of the blank-N is calculated out from the measured $\delta^{15}N$ –value of the sample (which contains both blank-N and target-N), thus removing the blank-N from the target N. After the blank correction the $\delta^{15}N$ values of the dilution serie should be linear.

Generally, the $\delta^{15}N$ of the blank is difficult to measure due to its small size. Thus, an arbitrary blank correction was performed. As mentioned above, no blank samples were included to the tested methods, and thus arbitrary area and $\delta^{15}N$-value were added to the Eq. 8. (mixing model) (see chapter 2.5.2 Blank samples and blank correction) for blank correction. The arbitrary blank correction resulted in more linear $\delta^{15}N$ values in each analysis except NH_4-

MD. As the N concentration increases, the influence of the blank becomes more negligible, as is shown in the Fig. 5. On average, two to three highest N concentrations (125, 250 and 500 µmol N L^{-1}) had linear $\delta^{15}N$ values after the blank correction. The averages (± standard deviation) of the two highest concentrations for NH_4-CM were depleted [-7.10 ± 0.45‰ and -8.01 ± 0.31‰, respectively]. Even in the two highest concentrations the NH_4-MD was not linear (5.71 ± 0.59 ‰ at 250 µmol N L^{-1} and -4.06 ± 0.74 ‰ at 500 µmol N L^{-1}) in the $\delta^{15}N$-values. The $\delta^{15}N$-values of NO_3-MD were close to zero in the two highest concentrations (and hence close to the reagent-NO_3^- value; 0.43 ± 1.19 ‰ at 250 µmol N $L^{-1}$and 0.93 ± 1.53 ‰ at 500 µmol N L^{-1}). When analyzed with CM the $\delta^{15}N$ values of NO_3^- of the two highest concentrations were enriched (6.06 ± 2.28 ‰ at 250 µmol N $L^{-1}$and 5.03 ± 2.34 ‰ at 500 µmol N L^{-1}).

4.4 Effect of the solution type and additional nitrogen species

The recovery of inorganic N dissolved in different solutions (milli-Q-H_2O and 1 M KCl for NH_4^+ analysis, milli-Q-H_2O only for NO_3^-) and impact of additional N species are shown in Fig. 6. The MD methods generally had higher recovery than CM with both NH_4^+ and analyses. Additionally, 1 M KCl solution produced higher recovery than milli-Q-H_2O for both methods when NH_4^+ was analyzed, but the difference was only significant for MD ($p < 0.001$). Excess N species showed no significant difference in recovery for CM, both NH_4-CM and NO_3-CM.

In contrast, NH_4-MD with extra N species produced significantly less N than solution without extra N ($p < 0.05$ against milli-Q-H_2O; $p < 0.001$ against 1 M KCl).

A significant difference was also found in the recovery percentages in NO_3-MD analyses: the additional N species caused highly significant increase ($p < 0.001$) of 26.9 %-unit in recovery, when compared to a solution containing only NO_3^-. On the contrary, NO_3-CM had a 0.2 %-unit higher recovery-% than with the additional N species (corresponding actually to 42.9 % increase) between averages. No significance, however, was observed in the recovery in CM method caused by the extra N species.

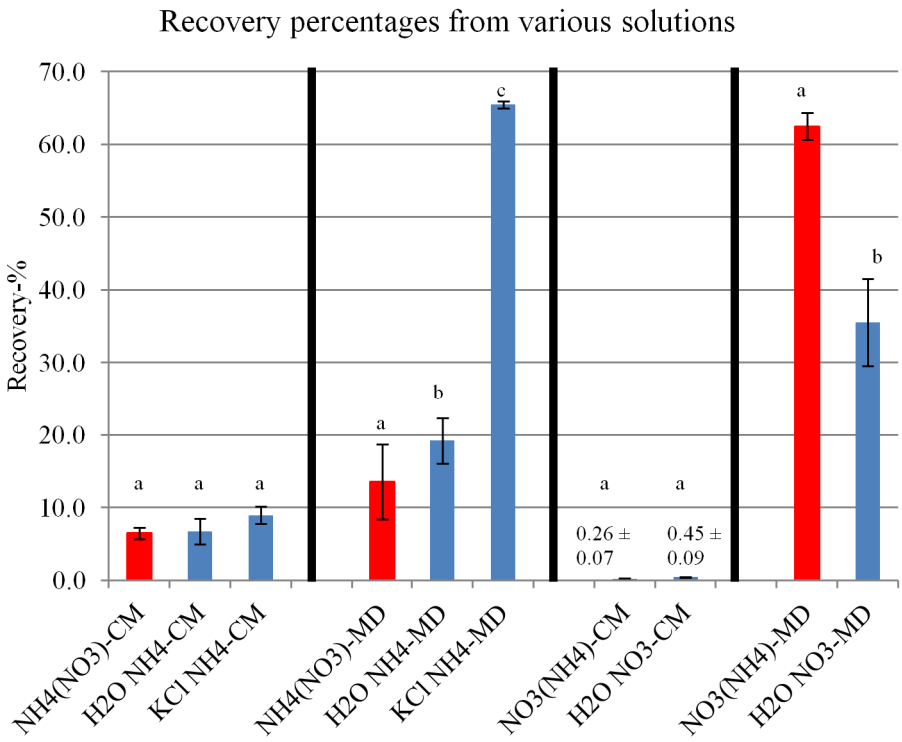

Figure 6. Recovery (in %) of the nitrogen components dissolved in different solutions and backgrounds (500 µmol N L^{-1}) for each used method; red bars indicate recoveries of samples containing excess N species which were made in milli-Q-H_2O solution. Black lines separate different methods, and the letters indicate significant differences with one-way ANOVA for the corresponding method. Note that KCl solution was used only for NH_4^+ analyses. Error bars indicate the standard deviation. Abbreviations: CM corresponds to chemical conversion method, MD corresponds to microdiffusion method, $NH_4(NO_3)$ and $NO_3(NH_4)$ corresponds to solution with both inorganic N (1st N is the target N while N-form in the parenthesis is the excess N species), KCl corresponds to solution with 1 M KCl and NH_4^+ and NO_3^- are only salts in the solution.

As shown previously and also in Fig. 7, generally the methods for the isotope analysis of NH_4^+ produced too negative $\delta^{15}N$ values while methods for NO_3^- produced too positive $\delta^{15}N$ values. If both methods are compared, MD produced closer $\delta^{15}N$ values to the source for both NH_4^+ and NO_3^- analyses than CM (Fig. 7).

Ammonium samples analyzed with CM had $\delta^{15}N$-value between - 8 and - 9 ‰. The excess N species and solution had no significant impact on $\delta^{15}N$-value in CM methods. While the use of 1 M KCl-solution increased significantly the recovery in NH_4-MD when compared to milli-Q-H_2O, no significant difference was found between their $\delta^{15}N$-values, which both were around 1.5 ‰. Unexpectedly, NH_4-MD containing excess N species had significantly more depleted (more negative) $\delta^{15}N$ value than solutions without excess N species ($p < 0.001$ for both 1 M KCl and milli-Q-H_2O) with a of $\delta^{15}N$ value of 3.5 ‰ on average.

Finally, both CM and MD methods differed significantly from the actual NH_4^+ source, which was analyzed to be 0.17 ± 0.46 ‰.

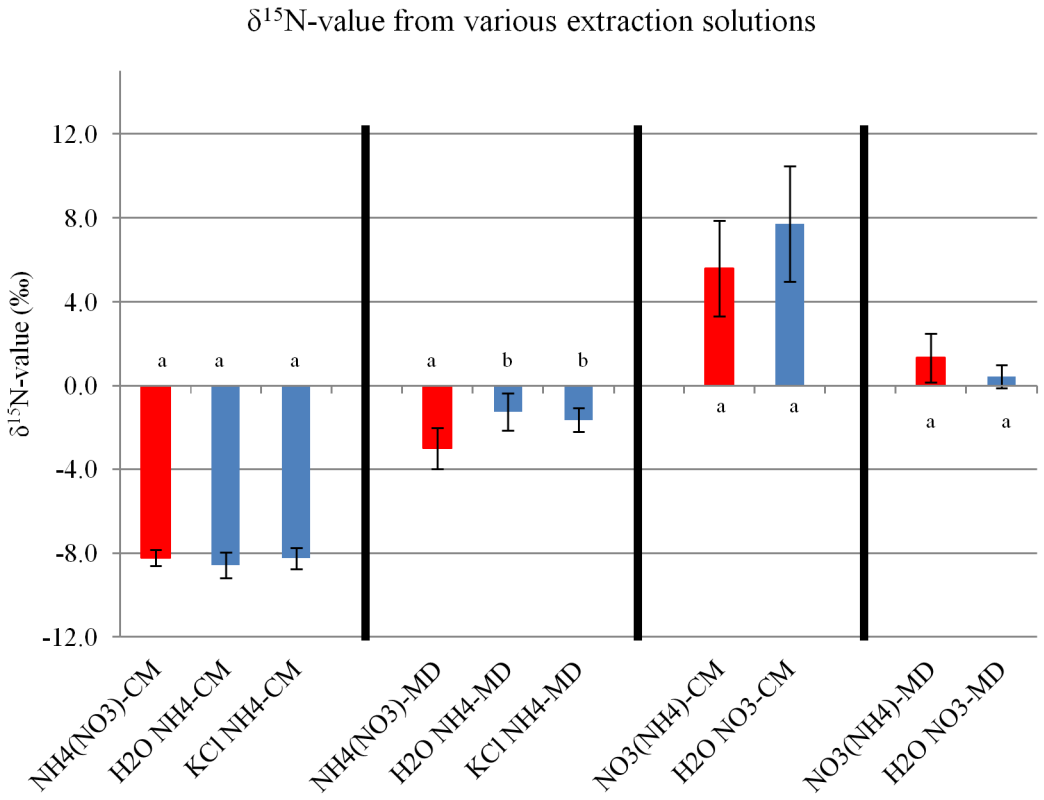

Figure 7. $\delta^{15}N$ values of nitrogen components dissolved in different solutions (500 µM N) for each method used; for abbreviations and color code, see legend of Figure 6.

The $\delta^{15}N$-values of NO_3^- showed no significant difference caused by excess N species, when analyzed with both methods. The $\delta^{15}N$-values of NO_3-CM methods ranged between of 8.13 ± 2.78 and 5.88 ± 1.99 ‰ and were thus significantly different from the source. On the contrary, the $\delta^{15}N$ values obtained from NO_3-MD had no significant difference to the actual NO_3^- source, which was analyzed to be -0.88 ± 0.27 ‰, and hence was the only method tested which provided the "correct" result.

5. DISCUSSION

5.1 Linearity of N concentration and recovery over the dilution series and the impact of solution type and excess N

Generally, the amount of N produced with both NO_3-CM and NO_3-MD showed good linearity over the dilution series. In contrast to NO_3^- analyses, NH_4^+ analysis showed an unexpected curvilinear behavior with both CM and MD. This curvilinearity was especially surprising for MD, since the N amount of both NH_4-MD and NO_3-MD was initially same, and the measurement protocol was also rather similar: NO_3^- is first reduced to NH_4^+ by Devarda´s alloy, from which form the protocol continues as with NH_4-MD. We can rule out problems with the Devarda's alloy, since also CM method showed this curvilinearity with NH_4^+. Thus, due to unclear reasons these differences in linearity between NH_4^+ and NO_3^- were seen. There may have been basic problems in the preparation of the NH_4^+ solutions, and other problems as further discussed below. The lack of sufficient blanks caused unrealistically high recoveries in the lowest concentrations for MD: concentrations of 7 – 15 and 7 – 30 µmol N L^{-1} ranged between 240 - 142 % and 333 - 123 % for NH_4-MD and NO_3-MD, respectively. Recoveries higher than 100 % were clearly derived from reagents used (blank), which were in our laboratory procedures unknown. Thus, the recoveries of the mentioned concentrations are not further discussed.

The MD with higher concentrations resulted with decreasing recoveries with increasing concentration (here 30 – 500 µmol N L^{-1}), dropping from 77 to 13 % in NH_4-MD. Microdiffusion of NO_3^- showed higher recoveries than NH_4-MD with increasing concentrations, ranging from 89 to 61 % for the concentrations of 60 – 500 µmol N L^{-1}, but as NH_4-MD, the recovery decreased against increasing concentration. Neither of MD analyses did reach ~100 % recovery as it should be for accurate analysis of isotope values – especially at natural abundance. Studies made by e.g., Stark & Hart (1996) and Stephan & Kavanagh (2009) provided blank-corrected recoveries of ~100 % in MD methods. A possible reason for decreased recovery was the diffusion time used in our procedure: while the recommendation is six to ten days (e.g. Stephan & Kavanagh 2009), we incubated samples for three days as recommended in the protocols adapted. Additionally, Stark & Hart (1996) reported recovery of > 96 % in NH_4-MD after six days of incubation. Hence, a likely reason for our reduced recovery was too short incubation time, with only half of the days recommended. Stephan and Kavanagh (2009) also suggested to use a small headspace (ratio of sample volume to vial volume) to achieve better recoveries.

The recoveries from the higher N-containing samples were at far too low range for MD, although containing the blanks of unknown magnitude. Stephan & Kavanagh (2009) made a detailed analysis of MD for both NH_4^+ and NO_3^- and their blank-N-sources: they found average contamination of 1.4 and 7.3 % of N originating from KCl to NH_4-MD and NO_3-MD, respectively. Additionally, MgO and Devarda´s alloy contributed 2.9 % on average to NO_3-MD. Thus, the NO_3-MD contained more than 10 % of blank-N in their study. Depending on the producer, KCl can be largely contaminated with N (Wolfgang Wanek, personal communication). In this study, the blank sources were responsible for the unrealistic high recoveries in the low concentration ranges and increased the recoveries, which were below 100%, in the high concentration ranges. As the blank samples were not done in our experiments, we assume that ~3 % of NH_4^+ recoveries and ~10 % of NO_3^- recoveries were most probably derived from the reagents, thus having high contribution – especially for NO_3-MD.

The use of 1 M KCl had a significant increase in recovery of 40 % in NH_4-MD compared to the milli-Q-H_2O. The use of KCl and other K-containing salts are known to increase the liberation of NH_3 in alkali solutions (pH > 9), as they bind the H_2O molecules and hence reduce the activity of H_2O (Mulvaney et al., 1997 and references therein). Efficient liberation of NH_3 from NH_4^+ pool and trapping of the NH_3-gas are the basis of the MD methods presented here. In the used laboratory procedures for method comparison, NH_4^+ dilution series was made to milli-Q-H_2O. Thus, the liberation of NH_3 was reduced due to the solvent, while NO_3^- dilution series was made to 2 M KCl. When NH_4-MD was made to 1 M KCl-solution, a significant recovery with more than three-fold increment was seen, when compared to NH_4-MD made to milli-Q-H_2O and – for comparison – the recovery was on similar range with NO_3-MD made to 2 M KCl. Problems associated with the lack of KCl could also explain the curvilinearity in NH_4^+ dilution series mentioned above, and surely explains that recovery for NH_4^+ was lower than for NO_3^- over the whole dilution series. Thus, the use of KCl had clear influence on the recovery in MD methods tested. In environmental samples, NH_4^+ is usually extracted from soil with KCl solution, and thus this problem should not exist.

Solutions used for each dilution series contained both inorganic N forms (NH_4^+ and NO_3^-), to simulate natural conditions where both inorganic nitrogen forms are abundant. A significant drop of ~25 % in recovery was seen in NO_3-MD, when the target N was analyzed without excess N species (NH_4^+), implying that the NH_4^+ was not completely removed during the four hour in the presence of MgO. Thus, it is crucial to incubate samples longer than four hours to

allow all liberation (purification) of NH_4^+. This has in principle only importance only for NO_3^- analysis, since NO_3^- is finally converted to NH_4^+ in the analysis where NH_4^+ background will be disturbing. However, an unexpected, significant but smaller difference was also seen in NH_4-MD with and without NO_3^- (Fig. 6), which remains unexplained as the NO_3^- should not impact on NH_4-MD.

The recoveries achieved by NH_4-CM method ranged from 6.6 to 1.8 % over the dilution series from 7 to 500 µmol N L^{-1}. Recoveries from 7 to 125 µmol N L^{-1} decreased logarithmically with the increasing concentration, while the two last concentrations (250 and 500 µmol N L^{-1}) showed increase. According to the method description by Hauck (1982) and Saghir et al. (1993), N_2O is only side-product in the conversion, whereas most of the target N is converted to N_2, with a recovery of 1.5 – 3.0 %. Our recovery was 1.8 – 6.6 %, thus slightly higher than the recovery of the method authors. A possible reason for this slightly higher recovery could be that in the original method the NH_3 released was first trapped to a cup with acid, from where it was further poured to a 12 ml vial prior the N_2O production. In our method, we trapped the NH_3 straight to the acid located in a 12 ml vial and hence avoided the pouring step, which might cause some sample losses to the cup walls.

Laughlin et al. (1997) provided an upgraded method for NH_4^+ conversion to N_2O, where the N_2O-recovery was increased up to 25 %. Our recoveries were clearly lower than 25 %. While Laughlin et al. (1997) allowed a diffusion time of four days prior the actual NH_4^+ conversion (with recovery of ~98 % as NH_4^+ -salt), our procedure had a diffusion time of only a day but with shaking (50 rpm). As the recovery of NH_4^+ -salt was not determined in our procedure, it is possible that the N recovered prior the conversion was already reduced. Additionally, tested 1 M KCl solution as solvent showed slight increase when compared to milli-Q-H_2O, but no significant increase was seen. However, 2 M KCl is recommended for solvent by the method description (e.g. Hauck 1982 and Laughlin et al., 1997), which was not tested.

Concentrations of NH_4^+ samples prepared with CM were also analyzed with GC-ECD and the results showed exactly same behavior as with results derived from Precon-GC-IRMS. When the concentration results of NH_4-CM from GC-ECD and Precon-GC-IRMS were plotted against each other, the R^2–value was 0.9975 (data not shown), indicating that the laboratory procedure as well as the solutions tested were similar as well as no instrumental problems occurred between different machines. Hence, the problem of NH_4^+ analysis seems to be related to NH_4^+ amount in the solution or to a dilution series made of it (see above discussion

concerning NH_4-MD). Taken into account the problems in the NH_4^+ linearity over the dilution series in all three different methods (that is NH_4-CM analyzed with both GC-ECD and Precon-GC-IRMS and NH_4-MD analyzed with EA-IRMS) there was most probably error in the standard dilution serie. However, as the original concentrations of NH_4^+ dilution series were not analyzed, this remains just speculation.

The chemical conversion of NO_3^- provided recoveries from 0.89 to 0.26 % over the dilution series of 7 to 500 µmol N L^{-1}, respectively. Steven & Laughlin (1994) showed ~10 % recovery from a sample containing 400 µmol N L^{-1} (the only recovery mentioned in their publication). Thus, the measured recoveries from NO_3-CM were 11 to 38 times lower than the expected range. Additionally, the mentioned recoveries still contain the unknown blanks and are thus even smaller. Original method description by Steven & Laughlin (1994) suggested to use so-called medical flat bottles (flat bottles with V = 108 ml) while we used infusion bottles (round bottles, V = 120 ml). In the laboratory step where the NO_3^- is converted to N_2O, the bottle is turned flat for the 2 h shaking, increasing the solution´s surface to headspace –ratio. The medical flat bottle provides much higher ratio than the infusion bottle, thus possibly enhancing the gas exchange between solution and headspace or decreasing it as in our case.

When extra N species (NH_4^+) was not present, the NO_3–CM provided an unexpected increase with almost doubling of the produced N_2O, though no significant difference was observed. As the reaction should not be sensitive to NH_4^+, the observed increase was most probably artificial.

5.2 $\delta^{15}N$ of nitrogen concentration over the dilution series as analyzed with different methods

The range of measured $\delta^{15}N$ –values were from +20 to -9 ‰ with all tested methods. Methods except NH_4-MD showed similar pattern in the raw $\delta^{15}N$-values over the dilution series: decreasing concentrations showed logarithmically increasing $\delta^{15}N$ –values. Similar to results on N concentrations, the measurements on $\delta^{15}N$–NH_4^+ from MD resulted in a curvilinear trend. Ideally, the $\delta^{15}N$ –values should provide a linear trend over the dilution series and this is mainly achieved after the blank-correction. However, as discussed in previous chapter (5.1 Linearity and recovery of N concentration over the dilution series and the impact of solution type and excess N), the blank samples were not included in this measurement protocol. Thus, the main problem, which resulted in non-stable $\delta^{15}N$ –values over the dilution series, lied in the lack of real blank correction.

The nitrogen background or blank results from traces of N found often in reagents used, contaminated laboratory equipment and/or inaccuracy in work procedures. Additionally, the machine background such as leakages in capillars like N_2 for Conflo-EA-IRMS-analysis and N_2O for Precon-GC-IRMS-analysis as well as compounds with the same mass number (e.g. CO has same mass with N_2; CO_2 has same mass with N_2O) interferes the accuracy. The combination of above-listed components can be measured as a blank sample by treating e.g. bare solvent - which was used for the target N analysis laboratory procedure - as a sample and thus "collecting" all possible contaminating N sources coming from different steps over the sample preparation and final analysis. The sample can finally be mathematically corrected from the blank result, both for concentration as well as for isotope values. Thus, to obtain accurate $\delta^{15}N$ –values of the target N, the blank has to be known both for its concentration as well for its $\delta^{15}N$ –value. In future applications of ^{15}N natural abundance, blanks have to obviously include in the measurement protocol. It has to be noted that impact of blank is most likely relatively small when labeled samples are analyzed, especially when concentration is high.

The microdiffusion of NO_3^- was the only tested method producing $\delta^{15}N$ –values comparable without significant difference to the actual salt used ($NaNO_3$). These consistent results were measured only with the two highest concentrations (250 and 500 µmol N L^{-1}). The decreasing concentrations produced logarithmically increasing $\delta^{15}N$ –values and the smallest concentration had a $\delta^{15}N$ –value of 7.9 ± 1.1 ‰. As discussed above, the results contain also the blank-N in which the exact $\delta^{15}N$ –value is unknown. The relative fraction of blank-N becomes more important with the decreasing target-N concentration. Thus, the two highest NO_3-MD concentrations were the only samples providing no significant difference to the target N with N amounts high enough to minimize the impact of blank-derived N. Finally, the results indicate that the concentrations starting from 250 µmol N L^{-1} with recovery of ~60 %, the NO_3-MD is sufficient for $\delta^{15}N$ –value determination at natural abundance level. However, the accuracy would have most probably been higher if the blank correction would have been done and higher recovery could have been achieved.

The decreasing concentrations produced enriched $\delta^{15}N$ –values, in which the enrichment was most likely derived from the blank. If the recoveries of the actual samples were less than 100 %, as they were with the four highest concentrations in NO_3-MD, the obtained results should have been depleted when compared to the actual source and hence be slightly negative. This assumption is based on the kinetic isotope fractionation theory (e.g. Fry, 2006), in which the

declining recoveries should have shown decreased (depleted) values, which were not measured. However, the $\delta^{15}N$ –values were slightly enriched, ranging from +7.9 to +3.3 ‰, respectively, in the low concentrations (7 to 125 µmol N L^{-1}), and recoveries were larger than 100 %. These results indicated that the blank was enriched in $\delta^{15}N$ and it significantly contributed to the amount of nitrogen recovered from the samples.

When NO_3-MD was tested for solution without excess N species (here NH_4^+), the $\delta^{15}N$-value tended to decrease but no significant difference was seen against the sample containing NH_4^+ as well. This was expected, as the $\delta^{15}N$-values of the original salts (NO_3^- and NH_4^+) were analyzed to be almost equal with each other. However, as mentioned above in previous chapter, a 25 % drop in recovery was observed in NO_3-MD when sample contained no NH_4^+. Thus, if NH_4^+ would have had clearly different $\delta^{15}N$-value, the contribution of 25 % would have significantly impacted on the $\delta^{15}N$-value as well. Thereby the proper NH_4^+ -removal is crucial for $\delta^{15}N$-NO_3^- analysis – at least at natural abundance levels. If NH_4^+ is not removed completely, it will be dissociated with reduced NO_3^- to the acid trap and hence the N pools are mixed (some NH_4^+ -originating N will be trapped as well as the target NO_3^- -N).

The microdiffusion of NH_4^+ produced unexpected $\delta^{15}N$ –values. Mid-concentrations of 60 – 250 µmol N L^{-1} ranged between +6.3 and +6.5 ‰, while three smallest and the highest concentrations produced depleted values (7 – 30, 500 µmol N L^{-1}) ranging between -3.4 and -6.9 ‰. Thus, no linear behavior was observed there, similar to recoveries. The NO_3-MD laboratory procedure followed exactly the procedure of NH_4-MD after the NO_3^- reduction to NH_4^+. As NO_3-MD showed both linear increase in dilution series as well as it provided the only $\delta^{15}N$ –values comparable to the actual salt, NH_4-MD seemed to have failed most likely due to mistakes in the preparation of the dilution series and is hence not discussed further.

Chemical conversion method of NO_3^- resulted in both raw data and corrected data in relatively high (enriched) $\delta^{15}N$ values, logarithmically decreasing from +20.2 to +5.9 ‰ with the increasing concentration. Hence, isotope values produced were clearly more enriched than the actual source ($NaNO_3$). The enrichment was most probably unavoidable since the target product of the method, N_2O, comes as a side product from a reaction between intermediate products with a yield of 10 % (Steven & Laughlin, 1994). Small recoveries of approximately 0.26 – 0.89 % were measured, as discussed in chapter 5.1. Most probably the kinetic isotope effect was strongly pronounced here, resulting in enrichment in ^{15}N content of N_2O, finally also producing the most enriched products of all of the tested methods. When

NO_3-CM was tested for solution without excess N species (here NH_4^+), the $\delta^{15}N$-value tended to increase but no significant difference was seen. However, the applicability of method producing such highly enriched values for the natural abundance analysis is scarcely practical – especially as other methods are known to be much more precise.

The $\delta^{15}N$ values determined with NH_4-CM results showed similar logarithmic decrease with concentration than NO_3-CM, except that the two lowest concentrations of the dilution series were lower in ^{15}N than the actual source. As with NO_3-CM, the produced N_2O in NH_4-CM was a side-product, which should – according to the kinetic isotope effect – be more enriched than the actual source. A possible reason for the depleted values could be the first step of NH_4-CM: NH_4^+ is first dissociated as NH_3 to acid in basic conditions. If that step would have been inefficient in the sense of recovery, then the dissociated NH_3 would be already depleted prior to the conversion to N_2O (Laughlin et al., 1997). However, we did not measure the actual recovery of the dissociation NH_3 to the acid, and thus can only speculate here.

No significant differences were observed in the $\delta^{15}N$ –value analyzed with NH_4-CM when tested in a KCl-solution, or when the solution contained an excess N (NO_3^-). Results indicate that the dissolution of NH_4^+ is not increased by the use of 1 M KCl, although Laughlin *et al.* (1997) suggests using 2 M KCl. Additionally, the reaction mechanism is independent of the existing NO_3^-.

As the blank samples were not included in the protocol, an arbitrary blank-correction was conducted with estimated values. “Random” values were tested in blank correction until best fit (or linear $\delta^{15}N$ values) was found. Generally, the arbitrary blank-correction provided the expected correction to each method (except NH_4-MD): the $\delta^{15}N$-values tended to stay linear over the dilution series and matched the actual $\delta^{15}N$-values derived from the original N source with higher precision. Only one to two smallest concentrations provided enrichment or depletion in $\delta^{15}N$-values. For example, NO_3-CM produced a range of +5.9 to +20.2 ‰ as uncorrected values for dilution series of 7 – 500 µmol N L^{-1}. The $\delta^{15}N$-values ranged there from +5.7 to +5.5 ‰ for dilutions of 30 – 500 µmol N L^{-1} after arbitrary blank-correction, respectively, thus having a difference of 0.2 ‰. The two smallest concentrations (7 and 15 µmol N L^{-1}) were -2.2 and -56 ‰, respectively. If fraction of blank is too high in the sample, e.g. in the smallest concentrations, then isotope results are not reliable anymore even if blank corrections are conducted.

Microdiffusion of NH_4^+ showed no difference between uncorrected isotope values and the blank-corrected ones (arbitrary blank correction). Thus, the problems occurred in NH_4-MD were most probably related to other issues than blank-size and its impact on the measured values for area or the $\delta^{15}N$-values, as discussed above.

Each of the methods tested included laboratory phases, where the target N was converted from a form to another (e.g., NH_4^+ oxidization to NH_3 and its dissociation to acid trap or to acid solution). Discrimination occurs in such phase as, e.g., the lighter (depleted) NH_4^+ molecule oxidizes faster to NH_3 than heavier ones, as well as the dissociation to the acid occurs faster to the lighter molecules than to the heavier ones (Fry, 2006). With increasing recovery the discrimination is of less importance (see Figure 1). Hence, if the initial conversion of N form to another form is insufficient, the converted N will be subjected to fractionation. Fractionation is additive, i.e. it accumulates over the whole reaction chains. We recognized such fractionation especially in CM methods, where N_2O is only a small side product. The MD methods produced generally enriched $\delta^{15}N$-values with decreasing concentration (except NH_4-MD). Most probably these enrichments were related to the unknown blanks, effects which seemed to override effects of fractionation in MD.

5.3 Future suggestions for $\delta^{15}N$-analysis of inorganic N at natural abundance

When a sample is either prepared for analysis or analyzed, it is important to obtain as high recovery as possible. Fractionation processes are obscuring isotope results if less than 100 % of the substrate is converted to the product. As for nitrogen analysis, in many cases a depletion of isotope values will be observed, due to the lower weight of ^{14}N. The limited recovery-% will cause then a result which does not match the substrate and is thus not reliable. Thus, high recoveries are prerequisites for correct analysis of isotopes by CM and MD methods.

Another important step which we identified in the method test for natural abundance samples was the blank correction. Without blanks the recoveries of MD´s with the smallest concentrations (both NH_4^+ and NO_3^-) were far above 100 %, while CM´s were less than 10 % at each case. Since blank samples were not done for neither of the methods tested, this indicates clearly the high background impact of the MD methods. Even if the blank analysis would be done, it still is a qualitative problem, especially with small N concentrations. Small inorganic N concentrations are often found from e.g. arctic soils, where most of the N – up to 94 – 99 % - is stored into organic matter, which is known to turn over very slowly due to long

cold season and high soil moisture content (Shaver et al., 1992). Thus, the analysis of small N concentrations and their isotopic N composition is matter of importance. Chemical method is clearly more reliable in the sense of low N amounts.

Both Zheng et al. (2007) and Lachouani et al. (2010) had emphasized the importance of the blank correction for the δ^{15}N-value by adding sets of known N standards to the sample batches, while latter authors added also a concentration series of a known N. Standards with different δ^{15}N-value (covering range of 30 ‰, e.g. -15 to +15 ‰) were necessity for both applications as the azide (N_3) brings one N atom to the produced N_2O. The standards with at least four different δ^{15}N-values and a dilution series (e.g. five different concentrations) in the latter author´s method were treated like samples and thus carried over the whole laboratory procedure.

Zheng et al. (2007) discussed the blank-N in NH_4-CM-analysis to be below the detection limit in their experiment and thus having no impact on the natural abundance levels. Similarly, Lachouani et al. (2010) argued of the problems in direct measurement of blank-N in their NO_3-CM due to the small N content in the blank. However, Lachouani et al. (2010) presented an indirect determination of both area (equals to amount) and δ^{15}N-value of blank-N based on the analyzed standard dilution series. They plotted the NO_3^- standard dilution serie on the x-axis against the corresponding signal intensities (in Vs) determined by Precon-GC-IRMS, in which the intercept of the linear regression corresponded to the blank-N area (in Vs). The δ^{15}N-value of the blank-N was determined by plotting the reciprocal (1/Vs) standard areas on x-axis and the corresponding δ^{15}N-values were plotted on y-axis. By inserting the calculated reciprocal blank intensity into the linear regression equation, the δ^{15}N-value of the blank can be calculated. Then, re-calculation of analyzed blank samples and standard dilution series proved to give accurate estimation of blank-N for both concentration and δ^{15}N-value and thus correction of analyzed samples. Additionally, accurate quantification of target N in excess to its δ^{15}N-value was possible by their method due to the high (104 ± 6 %) recovery.

In addition to blank-N information, by adding known standard dilution series to the sample batch – and carrying standards over the whole laboratory procedure – would provide instant information of inaccuracies occurring in the laboratory procedures, contaminated reagents or reduced recoveries. Thus, the addition of dilution serie would be critical future addition to the isotope analysis of N.

As discussed in previous chapter, Stephan & Kavanagh (2009) tested the N contributions of used reagents in the MD methods. As the reagents are known to contain small N amounts which interferes the accurate $\delta^{15}N$ analysis of a sample, they estimated the contributions of N contamination (amount N) derived by different reagents by so-called tracer method and the $\delta^{15}N$-values of the reagents by increased reagent amounts. The principle of tracer method was to measure the dilution of enriched N caused by reagents and thus calculate the amount of reagent-N; the dilution of enrichment could be provided only from lighter N (here reagent-N), derived from the reagents which are commonly having $\delta^{15}N$-values at natural abundance range. Second test made by Stephan & Kavanagh was to run much higher (up to 10 times) reagent amounts than the method recommendations are over the MD procedure and hence measure directly the $\delta^{15}N$-value of different N-containing reagents. After a detailed reagent analysis for their N-concentration and $\delta^{15}N$-composition, the same reagents can be used for a longer time.

6. SUMMARY AND CONCLUSIONS

Taken together, both methods produced $\delta^{15}N$ values which were, in most cases, significantly different from the source. Microdiffusion yielded to some extent better values than CM and can be given priority in the present method comparison. However, with respect to accuracy in $\delta^{15}N$ analysis of inorganic N at natural abundance level, each of the tested methods showed clear limitations and short-comings. As each of the methods is originally meant for studying of enriched samples, the revealed necessity of "fine-tuning" of the methods for natural abundance applications was not surprising. The lack of blank samples for each analysis caused severe problems for the isotope value correction. An artificial correction was conducted showing then the expected behavior over the dilution series in the different methods isotopic composition. Problems in NH_4^+ solutions used for each of the NH_4^+ experiments showed inconsistence in the dilution series. Thus, the NH_4^+ analyses were doubtful for their linearity and recovery and also for isotopic composition.

Microdiffusion has been used successfully for many years in our laboratory but only for the enriched samples provided from e.g. pool dilution experiments of NH_4^+ or NO_3^-. In such studies the enrichment level is often ranging within 1 – 15 AT-%, and with such enrichment levels the discrimination caused by e.g. reduced recovery or high blank-contamination does not cause severe impact in isotope accuracy, as they cause with natural abundance studies. However, both methods proved yet not sufficiently reliable for reliable measurements of inorganic N forms at natural abundance level. Some recommendations can be given to improve the results: 1) Prolongation of incubation time in smaller vessels would provide higher recovery. 2) The efficient NH_4^+ removal prior the NO_3-MD w as well as the presence of K-containing salts (e.g. KCl or K_2SO_4) in the solutions is important. Microdiffusion of NO_3^- was the only method providing $\delta^{15}N$-values equal to the original salt tested, although only at the two highest concentrations. Additionally, the dilution series made with NO_3-MD showed high linearity. Thus, the success in the NO_3-MD implies that this method was to some extent working. For comparison, the CM methods were tested for the first time in our laboratory. As N_2O is only a side-product in both CM methods, drifting $\delta^{15}N$-values were expected. The conversion of NO_3^- provided enriched values while NH_4-CM produced depleted values. Additionally, the NO_3-CM provided high linearity over the dilution serie. The discrimination constants could be settled for both CM, but more recent CM based on vanadium chloride reactions are shown to be much more precise than the tested CM.

The method limitations for accurate $\delta^{15}N$ analysis could be strongly enhanced by preparing a dilution serie of a known N and running them over the whole laboratory procedure. Thus the key problems found here – e.g. reduced recovery and problems with the blank samples – would be solved and corrected according to the very recent analytical and numerical methods, which were found during the experiment. The detailed reagent analysis would also progress the accuracy and reliability of the $\delta^{15}N$ analysis at natural abundance levels.

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APPENDIX

CHEMICAL REACTION BASIS OF THE EVALUATED METHODS

1. Microdiffusion of inorganic N (NH_4^+ and NO_3^-) as NH_4^+

Inorganic nitrogen (N) form [here (ammonium (NH_4^+) and nitrate (NO_3^-)] is reduced to gaseous ammonia (NH_3) in alkaline conditions in a closed vessel. Vessel contains so-called acid trap, in which the NH_3 is oxidized back to NH_4^+ -salt. Produced NH_4^+ -salt can be analyzed for its $\delta^{15}N$-content with EA-IRMS. Methods are adapted from Stark & Hart (1996)

1.1 Acid traps for N microdiffusion

Perforate filter paper with a paper puncher to gain filter paper discs. Cut 10 cm peace of teflon –tape and add two filter paper discs to the other end of the tape. Add 7.5 µl of 2.5 M $KHSO_4$ – solution on the discs and turn the other end of the tape over the discs. Seal the filter paper areas of the tape carefully with a circle object to keep the acidified filter papers free of water by pressing gently.

Reagents and equipment needed:

Ashless filter paper

2.5 M $KHSO_4$

Paper puncher

Teflon-tape

1.2 NH_4^+ microdiffusion

Ammonium is reduced in alkali conditions to NH_3, which is trapped in acid trap (see Appendix 1.2 Acid traps for N microdiffusion). The N-containing acid trap can be analyzed for its $\delta^{15}N$ with EA-IRMS.

Sample solution is first made alkali by magnesium oxide (MgO) addition.

Reaction 1. $MgO\ (s) + H_2O \rightarrow MgOH + OH$

$$NH_4\,(s) + OH \rightarrow NH_3\,(g) + H_2O$$

Magnesium oxide is hydrolyzed and hence the solution is turned alkaline (pH > 9.4). Ammonium is reduced to NH_3 (gas) in alkaline conditions, as the balance between NH_4^+ and NH_3 in alkaline conditions is completely on NH_3.

Reaction 2**.** $NH_3\,(g) + KHSO_4 \rightarrow NH_4\,(s) + KSO_4$

Ammonia is oxidized to NH_4^+ -salt by the acid within the acid trap, as the balance between NH_4^+ and NH_3 in acidic conditions is completely on NH_4^+.

After finishing the MD, the sample bottles are opened and the acid traps are collected, dried carefully and put into an open eppendorf vials, which are placed into a desiccator. A decanter glass with concentrated sulphuric acid (>97 % H_2SO_4) is used to dry the MD samples. After drying the samples are put to tin cups and samples are ready for ratio mass spectrometry (EA-IRMS) analysis.

Reagents used**:**

MgO

KCl

H_2SO_4

1.3 NO_3^- microdiffusion

Prior the NO_3-MD the existing NH_4^+ in the solution has to be removed. The removal is done by adding of MgO to the solution, and shaking the open bottle for 4 h (see Reaction 1.).

After NH_4^+ is removed from the sample, Devarda´s alloy and acid trap are added to the vessel, which is immediately sealed. NO_3^- is first reduced to NH_4^+, which - in alkali condition (pH > 9) - is further reduced to NH_3 which can be trapped with acid trap.

Reaction 3**.** NO_3 + Devarda-reagent $\rightarrow$ NH_4

After Reaction 3 the NO_3-MD proceeds as with NH_4–MD as described in 1.3 NH_4 microdiffusion, and thus NO_3^- is finally analyzed as NH_4^+.

Reagents used:

Devarda´s alloy

For other reagents and equipment used, see 1.3 NH_4^+ microdiffusion.

2. Chemical conversion of inorganic N (NH_4^+ and NO_3^-) to N_2O

Ammonium and NO_3^- have individual conversion methods to produce analyzable N_2O. Ammonium is first reduced to NH_3 in alkaline conditions, and then re-oxidized to NH_4^+ -salt. Adding NaOBr reduces NH_4^+ -salt to N_2 and N_2O, from which the latter can be analyzed for its $\delta^{15}N$ with Precon-GC-IRMS. Method is adapted from Hauck (1982) and Saghir et al. (1993).

Nitrate is first reduced to NO_2^-. After the addition of buffer solution and a Cd/Cu-reductor, the NO_2^- starts to further reduce, while simultaneously the reaction intermediates HNO_2 and NH_2OH react and produce N_2 and N_2O, from which the latter can be analyzed with Precon-GC-IRMS for its $\delta^{15}N$. Method is adapted from Steven & Laughlin (1994).

2.1 Principle of chemical conversion of NH_4^+ to N_2O

Ammonium is first reduced to NH_3 in the alkali conditions (see Reaction 1 above) by addition of MgO. Produced NH_3 is oxidized by acid solution to NH_4^+ -salt. By adding NaOBr-solution to the dried NH_4^+ -salt, NH_4^+ is reduced and is liberated as N_2 and N_2O.

Ammonia is oxidized by the acid within the vial and NH_4^+ -salt is produced, as the balance between NH_4^+ and NH_3 in acidic conditions is completely on NH_4^+.

Reaction 4. $NH_3 + H_2SO_4/CuSO_4*5H_2O \rightarrow H_2SO_4/(NH_4)_2CuSO_4$

Solution containing $H_2SO_4/(NH_4)_2CuSO_4$ is dried in oven for excess water removal, and dried NH_4^+-salt remains in the vial. Then the same vial is closed with a cap, evacuated and flushed with helium (He) gas to purify the headspace of other N-containing compounds, and He is left to vial at atmospheric pressure.

Reaction 5. $NaOBr + H_2SO_4/(NH_4)_2CuSO_4 \rightarrow N_2 + N_2O$

NaOBr oxidizes the NH_4^+ -salt and NH_4^+ is liberated mainly as N_2 but with small portion as N_2O, which can be analyzed with Precon-GC-IRMS for its $\delta^{15}N$.

Reagents and equipment used:

500 ml infusion bottles and septums

Shaker

Vials (16*100 mm) with septum caps

1 ml disposable syringes with long needles (20G x 2 ¾'')

20 ml syringe with a 26G x ½'' needle

20 ml syringe with a 19G x 1 ½'' needle

KCl

MgO

H_2SO_4

$CuSO_4$

NaOBr (NaOH, Br_2 and ice)

5mM H_2SO_4 : 0.5 mM $CuSO_4$ solution (1 l)

Pour at least 0.5 l of distilled water into a 1 l volumetric flask. Measure 26.65 ml of concentrated H_2SO_4 into the flask and fill the flask to the graduation line to achieve 0.5 M H_2SO_4.

Dilute 0.5 M H_2SO_4 to 5 mM by measuring 10 ml of 0.5 M H_2SO_4 into a 1 l flask. Dissolve 0.1248 g of $CuSO_4 \cdot 5\ H_2O$ in H_2SO_4 and fill the flask to the graduation line to achieve 5mM H_2SO_4 : 0.5 mM $CuSO_4$ solution (note that dissolving of $CuSO_4$ is exothermic reaction).

NaOBr (500 ml)

Dissolve 100 g of NaOH in 400 ml of distilled water in a 500 ml Erlenmeyer flask. Store in refrigerator overnight. If precipitate develops filter the solution through a GF/D glass fibre paper. In a fume hood put the flask in a container filled with crushed ice and add 16 ml of Br_2 over a period of 30 minutes. Do not allow the mixture to reach temperature over 5 °C to prevent the competing reaction of bromide and bromate production. When all of the Br_2 has reacted, dilute to 500 ml with distilled water in a volumetric flask and store in a refrigerator. The reagent stands for several weeks in 4 °C.

2.2 Principle of chemical conversion of NO_3^- to N_2O

The method is based on the reaction between nitrite (NO_2^-) and hydroxylamine (NH_2OH) to form N_2O. At pH 4.7 NO_3^- is reduced to NO_2^-, NH_2OH and finally NH_4^+. Nitrous oxide is formed when the two intermediates (NO_2^- and NH_2OH) react. Nitrite present in the sample must be removed prior the NO_3^- reduction, as it would be added to the results targeting NO_3^-.

Reaction 6. $NO_2 + NH_2SO_3H \rightarrow N_2$

Decrease of pH to 1.7 reduces the existing NO_2^- to N_2, which is released to the atmosphere.

Reaction 7. $CH_3COONa{:}CH_3COOH$ + Cu/Cd(SO_4)-reductor →

$NO_3 \rightarrow HNO_2 \rightarrow NH_2OH \rightarrow NH_4$

Alkalinity is increased to 4.7 by buffer solution ($CH_3COONa{:}CH_3COOH$). The increment of pH stops the reduction of HNO_3 to N_2, and the Cd/Cu-reductor reduces the target NO_3^- via intermediates to finally NH_4^+.

Reaction 8. $HNO_2 + NH_2OH \rightarrow N_2O + 2H_2O$

Intermediates react and produce N_2O, with highest concentration gained after two hours of reaction time. Thus, after 2 h the N_2O sample is taken with a syringe to an evacuated vial, from which the N_2O can be analyzed with Precon-GC-IRMS for its $\delta^{15}N$.

Reagents and equipment used:

150 ml infusion bottles with septums

Vials (16*100 mm) with septums

Shaker

Gas tight syringe with a valve and needles (26G x ½'')

0.2 M NH_2SO_3H

CH_3COONa

Glacial CH_3COOH, 17 M

$CuSO_4$

Concentrated HCl

Cadmium foil 0.25 mm thick, 99.99 % purity (we have 0.5 mm thick, 99.85 % purity)

0.2 M sulphamic acid solution (100 ml)

Dissolve 1.9418 g of NH_2SO_3H in distilled water in a 100 ml volumetric flask. Fill up with distilled water to the graduation line.

1 M acetate buffer solution (1000 ml)

Dissolve 136.08 g of $CH_3COONa \cdot 3H_2O$ in distilled water in a 1l volumetric flask. Add 57.19 ml of glacial CH_3COOH and fill up with distilled water to the graduation line.

0.04 M $CuSO_4$ solution (1000 ml)

Dissolve 9.9872 g of $CuSO_4 \cdot 5\ H_2O$ in distilled water in a 1 l volumetric flask. Fill up with distilled water to the graduation line.

6 M HCl (1000 ml)

Add approximately 0.5 l distilled water in a 1 l volumetric flask. Add 497 ml of concentrated (37 % by weight) HCl and fill up with distilled water to the graduation line.

Cd/Cu reductor

Cut foil into 25x25 mm rectangular pieces. Form cylinders (reductors) with height of 25 mm and diameter of 8 mm. Treat Cd-reductors with 6 M HCl for 1 minute (turn a few times with forceps). Decant the HCl and wash the reductors thoroughly with distilled water. Treat reductors with 0.04 M $CuSO_4$ for 1 minute and wash them thoroughly with distilled water. Store the reductors in distilled water in a closed container.